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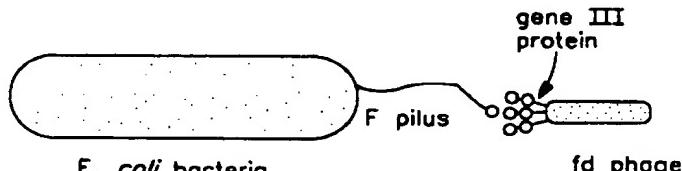
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(54) Title: A METHOD FOR DISPLAYING PROTEINS

(57) Abstract

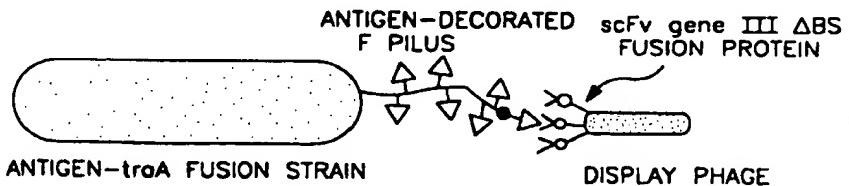
NORMAL INFECTION

The present invention relates to a fusion protein, comprising a pilin protein or a portion thereof and a heterologous polypeptide (target protein). In a preferred embodiment it relates to a method for displaying the target protein on the outer surface of a bacterial host cell capable of forming pilus. In certain embodiments, it is desirable that the pilus is a receptor for bacteriophage attachment and infection. The F pilus is preferred.



- 1) BINDING OF PHAGE TO THE TIP OF THE PILUS
- 2) DEPOLYMERIZATION OF PILUS
- 3) ENTRY OF THE PHAGE INTO THE CELL

ANTIGEN/ANTIBODY DRIVEN INFECTION



NORMAL GENE III PROTEIN/PILIN INTERACTION IS  
REPLACE BY ANTIGEN/ANTIBODY INTERACTION

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## A METHOD FOR DISPLAYING PROTEINS

The present invention relates generally to the exportation and display of polypeptides and proteins on the surface of bacteria. Methods are disclosed providing for display, modification, selection and purification of proteins, including antigenically active proteins, specific binding proteins  
5 and enzymatically active proteins.

## BACKGROUND OF THE INVENTION

The expression of polypeptides on the surface of bacteria and  
10 bacteriophage has been pursued for several years, in part because of interest in recombinant antibody production. Many other potential applications exist, including the production of genetically-engineered whole cell adsorbents, construction of "peptide libraries", cell bound enzymes, and use as live vaccines or immunogens to generate antibodies. [See,  
15 WO92/01047 and WO93/10214.]

In bacteria, one approach to obtaining surface expressed foreign proteins has been the use of native membrane proteins as a carrier for a foreign protein. In general, most attempts to develop methods of  
20 anchoring proteins on a bacterial surface have focused on fusion of the desired recombinant polypeptide to a native protein that is normally exposed on the cell's exterior with the hope that the resulting hybrid will also be localized on the surface. However, in most cases, the foreign protein interferes with localization, and thus, the fusion protein is unable to  
25 reach the cell surface. These fusions either end up at incorrect cellular locations or become anchored in the membrane with a secreted protein

domain facing the periplasm [Murphy, *et al.*, *J. Bacteriol.*, 172:2736 (1990)].

Francisco, *et al.*, [*Proc. Natl. Acad. Sci.*, 89:2713 (1992)] reported 5 constructing a surface-expression vehicle consisting of the lpp N-terminal targeting sequence fused to a sequence derived from *ompA* leaving the C-terminus exposed on the external side of the outer membrane. These fusions have been reported to export a number of heterologous proteins to the *E. coli* surface, including  $\beta$ -lactamase, single-chain Fv antibody and a 10 cellulose binding protein [W093/10214]. In addition, Fuschs, *et al.*, [*Bio/Technology*, 9:1369 (1991)] reported that a fusion between the *E. coli* peptidoglycan-associated lipoprotein (*pal*) and a lysozyme-binding single-chain Fv antibody fragment could be detected on the surface of bacteria. However, in these systems, the displayed proteins were affixed to the cell 15 surface, and thus in order to isolate purified protein, the DNA encoding the protein must be subcloned to another system.

Systems have been developed for displaying recombinant proteins, including antigens and antibodies, on the surface of filamentous 20 bacteriophage [see, for example, W092/01047]. In these systems, the recombinant protein is fused to the phage coat proteins expressed by either gene III (minor coat protein) or gene VIII (major coat protein). The display phage can be selectively enriched based on the binding properties of the recombinant protein. In addition, the phage carries a vector for 25 expression of the recombinant protein-gene III fusion allowing propagation of the display phage. One of the advantages of this system is that a large library of different proteins such as Fab or single-chain Fv antibody fragments can be displayed on the phage and selected for on the basis of their binding characteristics. One disadvantage is that the number of

heterologous protein molecules displayed by the phage is low, thus complicating the selection process. Another disadvantage with phage systems, as well as current bacterial systems is that the enrichment or panning process requires a significant amount of purified binding protein,  
5 e.g., antigen, and involves repeated rounds of selection and re-amplification that may result in the isolation of recombinant proteins, e.g., single-chain antibodies, with low binding affinities.

10 A display system combining the benefits of bacterial display and phage display has yet to be developed. Such a system would be very desirable.

15 It would also be desirable to have a method that can be used for cloning and protein purification without the need for subcloning.

It would be desirable to have a display and selection method that eliminates the need for panning and purification of binding protein.

#### SUMMARY OF THE INVENTION

20 The present invention relates to a fusion protein, comprising a pilin protein or a portion thereof and a heterologous polypeptide (target protein). In a preferred embodiment it relates to a method for displaying the target protein on the outer surface of a bacterial host cell capable of forming  
25 pilus. In certain embodiments, it is desirable that the pilus is a receptor for bacteriophage attachment and infection. The F pilus is preferred.

The fusion protein is expressed from a chimeric DNA having a DNA segment encoding a leader amino acid sequence capable of mediating

secretion of the fusion protein, a DNA segment encoding pilin subunits, e.g., the traA gene product, and a DNA segment encoding the target protein. The DNA segments are positioned such that expression of the fusion protein results in display of the target protein on the surface of the pilus. The pilus is preferably anchored to the cell surface of a bacteria forming what is referred to as a "display bacteria."

The chimeric DNA may be integrated into the bacterial cell chromosome or be carried by a vector. In certain preferred embodiments, 10 expression of the fusion protein may be regulated by an inducible promoter, e.g., lac. Bacteria displaying a particular protein may be selected, for example, using antibody affinity. The fusion protein can be detached from selected cells. If desired, the target protein may be separated from the pilin protein and further purified.

15

The present invention further relates to a method for selecting and isolating specific binding pairs, e.g., antigen-antibody, receptor-ligand. In accordance with this method, a display bacteria is formed in which one protein of the specific binding pair is displayed and replaces the natural 20 receptor for bacteriophage infection. The phage is also altered such that the normal pilin interaction domain is substituted with the other member of the specific binding pair or a library of proteins containing potential binding members. Alternatively, the bacteria display a library of protein containing potential binding proteins. The display phage is then contacted with the 25 display bacteria. Phage displaying one member of the specific binding pair recognize and infect the display bacteria displaying the other member based on the protein-protein interactions between the displayed proteins. The phage genome is then internalized by the display bacteria. These bacteria can then be selected by, for example, identifying of a marker

- gene, i.e., antibiotic resistance, transferred from the phage to the display bacteria. In addition, phage displaying high affinity binding proteins infect and replicate at a higher rate than the phage displaying lower affinity binding proteins. This allows phage displaying a library of potential binding 5 proteins to be screened for high affinity binding since these phage will be selectively enriched with continued growth in cultures of the display bacteria. DNA encoding members of the specific binding pair can then isolated from the display bacterial host.
- 10 As used herein, bacteriophage also include phage rescued from an *E. coli* host carrying a phagemid vector encoding the fusion protein. While such phage are capable of infecting the display bacteria, since they lack the necessary phage genes they cannot produce particles for reinfection and thus cannot be used in method where reinfection is desired.
- 15 In one embodiment, the DNA encoding a member of a specific binding pair is mutagenized, e.g., by use of a mutator strain, and the method of the present invention used to select a member of a specific binding pair having an altered binding affinity, e.g., increased affinity. In 20 another embodiment, compounds can be tested for their ability to affect, e.g., inhibit or potentiate, the specific binding pair interaction.
- Target proteins useful in the present invention include peptides, 25 proteins, e.g., hormones, enzymes, inhibitors, and receptors, antigens, antibodies including antibody fragments, single-chain antibodies and a member of a specific binding pair. Alternatively, the target protein may be a derivative or analog of any such proteins. Specific binding pairs include any pair of molecules, either naturally derived or synthetically produced in which one of the pair has an area which binds to the other molecule.

Examples of such specific binding pairs include, for example, antigen-antibody, hormone-hormone receptor, receptor-ligand, enzyme-substrate and IgG-protein A.

- 5        Other uses for the protein display methods of the present invention include, for example, epitope mapping, screening of antibody libraries and live bacterial vaccines.

**BRIEF DESCRIPTION OF THE DRAWINGS**

10

Figure 1 is a diagram showing the features of the traA expression vector pPR35.

15

Figure 3 sets forth oligonucleotides (SEQ ID NOS: 1-16) used in PCR amplification and vector construction. Relevant restriction sites are underlined.

20

Figure 4 sets forth the nucleotide (SEQ ID NO:17) and amino acid sequence (SEQ ID NO:18) of the pPR35 traA fusion region.

Figure 5 sets forth the construction of pGH21.

25

Figure 6 demonstrates the detection of antigen on the surface of a bacterial host cell by colony immunoblotting.

Figure 7 is a graph demonstrating antigen tag expression on the cell surface.

- 7 -

Figure 8 is a graph demonstrating recombinant antibody expression on the cell surface.

5       Figure 9 is a colony blot showing detection of anti-CK-MB activity of the cell surface.

Figure 10 shows the fd gene III protein structure function analysis.

10      Figure 11 illustrates the bacteriophage/pilin interaction system.

Figure 12 shows the recombinant protein/gene III-pΔBS fusion region.

15      Figure 13 shows the scheme for constructing the r-protein/gene III pΔBS display phagemid. The Oligonucleotides used in cloning are set forth below:

OPR1 - 5'-GGG GGG AGC TCT CTG CAA AGG AGA CAG TCA TAA TGA AAT ACC TAT TGC CTA CGG CAG CCG CTG GAT TG-3' (SEQ ID NO:19)

20      OPR2 - 5'-GGG GGG CCG CGG CCG CGG CCA TGG CCG GCT GGG CCG CGA GTA ATA ACA ATC CAG CGG CTG CCG TAG-3' (SEQ ID NO:20)

25      OPR3 - 5'-GGG GGG CCG CGG CCG CGG AGG AAG AAG AGT ACA ACC CGA ACG AAG GCG CCG CCT AGA CTG TTG AAA GTT GTT TAG CAA AAC CTC-3'(SEQ ID NO:21)

OPR4 - 5'-GGG CCG AAT TCC TAT TAA GAC TCC TTA TTA CGC AGT ATG TTA GC-3' (SEQ ID NO:22)

30      OGH1 - 5'-GGG GGG ACT AGT GCG GCC GCG GGC GCC GCT GAA ACT GTT GAA AGT TGT TTA GC-3' (SEQ ID NO:23)

OGH107 - 5'-GGG GGG GGA TCC AGA GGG TTG ATA TAA GTA TAG CC-3' (SEQ ID NO:24)

Figure 14 shows the construction of the r-protein/gene III p $\Delta$ BS display phage vector.

Figure 15 shows a Western blot analysis of partially purified TA-1 scFv/EE tag/traA fusion protein. XL1-B/pGH21 cells (1L) were grown and TA-1/EE tag/traA fusion protein expression was induced with IPTG as described in example 2. The bacteria pilin protein was partially purified by shearing the pili from the cells and PEG precipitation as described by Moore et al. (*J. Bacteriology*, 146 (1):251-259, 1981). The fusion proteins present in the induced cells (cell lysate lane) and in the partially purified protein (PEG ppt. lane) were examined by western analysis using the anti-EE tag mAb-HRP as a probe. the band corresponding to the TA-1/EE tag/traA fusion protein is indicated. The immunoreactive material at the top of the stacking gel is aggregated fusion protein that does not enter the resolving gel.

#### DETAILED DESCRIPTION OF THE INVENTION

F pili are filaments found on the surface of cells carrying the F plasmid. They are essential for establishing competent mating pairs during bacterial conjugation [see, Ippen-Ihler and Minkley, *Ann. Rev. Genet.*, 20:593-624 (1986)] and are the site of attachment for three classes of bacteriophage, R17, QB, and fd [Paranchych, Cold Spring Harbor Laboratory, pp. 85-111 (1975)]. The top of the pilus is thought to be involved in the recognition of a recipient cell (mating pair formation) or another donor cell (surface exclusion) and is the site of attachment of the filamentous phage, fd. The sides of the pilus are the site of attachment of two types of spherical phages, exemplified by R17 and QB.

Synthesis of the F pilus requires 13 or more gene products encoded by the transfer region on the F plasmid [Ippen-Ihler and Minkley, *supra* (1986)]. The F pilus is composed of a single subunit of 7,200 daltons encoded by the *traA* gene. The initial *traA* gene product is propilin  
5 (13,200 daltons) which contains 51-amino acid leader sequence. The pilin subunit is acetylated at the amino-terminus and *traG* is thought to be involved in this process [Ippen-Ihler and Minkley *supra* (1986); Willetts and Skurray, *American Society for Microbiology*, 2:1110-1133 (1987)].  
  
10 F-like plasmids encode four known types of pili which can be distinguished serologically [Lawn and Meynell *J. Hyg.*, 68:683-694 (1978); Meynell International Conference on Pili, pp. 207-234 (1978)], by phage sensitivity patterns or surface exclusion [Willetts and Maule, *Genet. Res.* 47:1-11 (1986)]. These pili are also thought to recognize different  
15 receptors on the surface of the recipient cell [Havekes, et al., *Mol. Gen. Genet.*, 155:185-189 (1977)]. Representative pilin genes from four types have been sequenced (Frost, et al., *J. Bacteriol.*, 164:1238-1247 (1985)) and the changes in protein sequence are found in the amino-terminus [Finlay, et al., *J. Bacteriol.*, 163:331-335 (1985)], with the carboxy-  
20 terminus influencing the antigenicity of the protein [Frost, et al., *supra* (1985)]. The four pilus types vary in their ability to attach to F-specific phages [Meynell, *supra* (1978)], which reflects changes in pilin sequence. However, the amino-terminus does not seem to be involved in phage attachment since pili with different amino-terminal attach fd phage equally  
25 [Frost, et al., *supra* (1985); Finlay, et al. *J. Bacteriol.*, 168:990-998 (1986)]. The changes in sequence which probably affect phage binding occur at residues 11 and 14 in type IV pilin (represented by the R100-1 plasmid) and at the carboxy-terminus in Type III pilin (represented by the R1-19 plasmid). Studies with polyclonal antisera [Worobec, et al., *J.*

- 10 -

*Bacteriol.*, 167:660-665 (1986)] and monoclonal antisera [Frost, et al., *J. Bacteriol.*, 168:192-198 (1986)] have shown that the major epitope at the amino-terminus is exposed in a tip-specific manner at the end of the pilus. The minor epitope(s) which involve the carboxy-terminus of the pilin 5 protein are exposed on the sides of the pilus.

The method of the present invention relates to displaying a heterologous polypeptide (target protein) on the outer surface of a bacterial host cell. This method comprises expression of a fusion protein, 10 comprising a pilin protein or a portion thereof and the target protein in a bacterial host cell capable of forming a pilus. The fusion protein being expressed from a chimeric DNA having a DNA segment encoding a leader amino acid sequence capable of mediating secretion of the fusion protein, a DNA segment encoding the pilin protein and a DNA segment encoding 15 the target protein, said DNA segments being operably linked such that the host cell displays the target protein on its surface.

Any bacterial strain capable of forming a pili can be used as a bacterial host cell for the expression of the chimeric DNA. Strain capable 20 of forming an F or F-like pili are preferred. Such strains include *E. coli* (Ippen-Ihler, et al.), *Salmonella typhimurium* [Artz, S. Holzschu, D., Blum, P., and Shand, R. (1983) Gene 26, 147-158], as well as other gram-negative bacterial carrying F-like plasmids. *E. coli* is the preferred host cell. Particularly preferred *E. coli* strains include XL1B [Bullock, W.O. et al. 25 (1987) Bio/Techniques 5, 376-378] and DH5 $\alpha$ F' [Woodcock, D.M. et al. (1989) Nucleic Acids Res. 17, 3469-3478]. In certain embodiments, *E. coli* strains that overexpress pili are preferred. Such strains include, for example, those that carry the depressed F-like plasmid pED208 [Frost, L.S., et al., (1985) J. Bacteriol. 164, 1238-1247].

The first component of the chimeric DNA is a DNA segment encoding a leader amino acid sequence capable of mediating secretion of the fusion protein, i.e., directing the fusion protein to the external membrane surface. Such sequences include, for example, the traA leader sequence, the phoA leader or the pelB leader. The traA leader sequence is preferred. The traA leader sequence may be obtained by PCR amplification from an F plasmid template. F plasmids are available, for example, from bacterial cells such as *E. coli* XL1B. A representative traA leader sequence is set forth in Figure 4.

10

The second component of the chimeric DNA is a DNA segment encoding the pilin protein subunit or a portion thereof capable of displaying the target protein on the cell surface. Mutation analysis suggest that the region of the pilin subunit between amino acids 18 to 68 contain elements required for pilus assembly (Frost et al., Mol. Gen. Genet. 213:134-139 (1988)). The traA gene product is preferred.

Hydropathy profiles the F-pilin suggests that the molecule is organized into four domains [Paiva, W.D., et al., (1992) J. Biol. Chem. 267, 26191-26197]. Variability in the number and type of amino acids present in the N-terminal domain is observed for different F-like pilin proteins [Frost, L.S. et al (1985)], suggesting that this region may be dispensable for pili assembly and display of on the cell surface. As described above, the traA gene encodes the 51 amino acid pilin leader and the 70 amino acid mature pilin protein. TraA genes have been cloned and sequenced from F and a number of related F-like plasmids, including ColB2 (Group II), R1-19 (Group III), R100-1 (Group IV), and pED208 (Group V)[Finlay, B.B. et al, (1984) J. Bactriol. 160:402-407; Frost, L. S. et al (1984) J. Bacteriol. 160:395-401; Frost, L. S. et al (1985); Finlay, B. B. et

al (1986) J. Bacteriol. 168:990-998]. These genes show a high homology with each other and encode pilin proteins that comprise morphologically and functionally similar structures, as emphasized by the formation of mixed pili by cells carrying different F-like plasmids [Lawn, A.M., et al 5 (1971) Ann. Institute Pasteur 120:3-8]. Since the sequences of various traA genes are available, the DNA encoding the traA gene product can be readily isolated from a number of sources, including for example, PCR amplification from an F plasmid template. See, Example 1 for the details of the PCR amplification. A representative F plasmid traA gene sequence 10 is set forth in Figure 4.

Target proteins, encoded by the third component of the chimeric DNA, can include peptides, proteins, e.g., hormones, enzymes, inhibitors, and receptors, antigens, antibodies including antibody fragments (e.g., Fab, 15 Fab' and F(ab')<sub>2</sub>) single-chain antibodies and a member of a specific binding pair. Alternatively, the target protein may be a derivative or analog of any such proteins. Specific binding pairs include any pair of molecules, either naturally derived or synthetically produced, in which one of the pair has an area which binds to the other molecule. Examples of such specific 20 binding pairs include, for example, antigen-antibody, hormone-hormone receptor, receptor-ligand, enzyme-substrate and IgG-protein A.

The nucleotide sequence of many target proteins are readily available through a number of computer data bases, for example, GenBank, EMBL 25 and Swiss-Prot. Using this information, a DNA segment encoding the desired target protein may be chemically synthesized or, alternatively, the such a DNA segment may be obtained using routine procedures in the art, e.g, PCR amplification.

The DNA segments are positioned such that expression of the fusion protein results in the display of the target protein on the cell surface, forming what is referred to as a "display bacteria."

- 5        The target protein may be fused to any portion of the pilin protein that is capable of displaying the target protein on the cell surface. Fusion to the amino terminal region of the pilin protein is preferred.

Successful display of the target protein on the cell surface can be detected using a number of methods, for example, if the target peptide can be specifically labelled by a procedure that does not operate through the membrane, its cell surface display can be readily demonstrated. This can be done by iodination ( $^{125}\text{I}$ ) of tyrosyl residues in the presence of lactoperoxidase [Marchalonis, et al., *J. Biochem.*, 124:921-927 (1971); King and Swanson, *Infect. Immunol.*, 21:575-584 (1978)].

In addition, one can examine if the target polypeptide is accessible to proteases added from the outside to intact cells. The action of the protease can be monitored by looking at the cleavage of the polypeptide by SDS-PAGE, or by examining if other properties of the polypeptide are affected (enzyme activity, antigenicity, etc.).

If the target polypeptide displays enzymatic activity, one may use such activity to demonstrate cell surface display. This can be done if a substrate unable to cross the outer membrane is available: nitrocefin is such a substrate for  $\beta$ -lactamase [O'Callaghan, et al., *Res. Microbiol.*, 141:963-969 (1972); Kornacker and Pugsley, *Mol. Microbiol.*, 4(7):1101-1109 (1990)]. It is important to ensure that the outer membrane is indeed impermeable to the substrate when the hybrid protein is expressed.

Antibodies against the target protein may also be used. However, these methods have limitations. First, the fusion protein may be constrained in conformation where the target polypeptide is not detected by the antibody used [Charbit, et al., *Embo J.*, 5:3029-3037 (1986); 5 MacIntyre, et al., *J. Biol. Chem.*, 263:19053-19059 (1988)]. Second, if the antibody is targeted to a short peptide within the target (for example, an epitope included within 10 residues), the results will only give information on this epitope; thus a positive result may indicate that only this short peptide is exposed, whereas a negative result may indicate that 10 part of the epitope is not accessible, which does not mean that some other part of the target protein is not exposed.

Binding of the antibodies to the bacteria can be examined with a number of different techniques. Such methods include, bacterial 15 agglutination, immunofluorescence, ELISA with intact cells, RIA with intact cells, immunoelectron microscopy, and targeted action of complement [see, M. Hofnung, *Methods in Cell Biology*, 34:77 (1991)].

The chimeric DNA may be integrated into the host cell chromosome 20 or be carried within a vector. Methods of integrating DNA into a host cell chromosome are well known in the art and include, for example, homologous recombination. See, Winona, et al. *J. Bacteriol.* 161:219-21 (1985). The chimeric DNA may also be carried within a recombinant vector, e.g., a plasmid. Recombinant vectors are preferred.

25

The recombinant vectors of the present invention comprise a vector backbone and the chimeric DNA. The recombinant vectors may include an inducible promoter sequence operably linked to the chimeric DNA. Promoters are well known in the art and can readily be selected depending

on what cell type is to be used for expression of the fusion protein. The DNA segment encoding the leader is preferably positioned downstream of the promoter sequence. The traA leader sequence is preferred. The DNA segment encoding the target peptide is positioned downstream of the 5 leader sequence. The DNA segment encoding the traA gene product is preferably positioned downstream of the DNA encoding the target peptide.

Plasmids useful as the vector backbone include plasmids containing replicon and control sequences which are derived from species compatible 10 with the host cell. For example, if *E. coli* is used as a host cell, plasmids such as pUC19, pUC18 or pBR322 may be used.

Vectors can also be constructed comprising the traA leader DNA segments and the traA DNA segment with a cloning site incorporated 15 between the DNA segments to allow insertion of DNA encoding a target protein or insertion of a DNA library. The vector may also contain an inducible promoter and marker gene, e.g., antibiotic resistance.

A preferred recombinant vector of the present invention is plasmid 20 pPR35. This plasmid contains a traA leader DNA segment and a traA DNA segment downstream of the inducible *lacZ* promoter of pUC19. Cloning sites for *Ncol*, *Sfil* and *Notl* are incorporated between the traA leader and traA protein sequences to allow insertion of the DNA segments encoding the target peptide. In addition, a DNA sequence encoding the EE tag 25 antigen is positioned between the traA leader and traA protein sequences to allow for detection of the fusion protein and characterization of the expression-display system.

Introduction of the chimeric DNA to the host cell may be effected by any method known to those skilled in the art. For example, if the DNA is carried by a recombinant vector, the vector can be introduced, for example, by transformation, electroporation, or phage transfection.

5

The detection techniques noted above can be used initially to verify that the method of the present invention is working, i.e., that the fusion pilin protein has been expressed and transported to the bacterial cell surface and is orientated so that the target protein is accessible i.e., 10 displayed.

Cells that display the target may be separated from those which do not, using, for example, affinity separation techniques. Such techniques include affinity column chromatography, batch elution from affinity matrix 15 material and fluorescent-activated cell sorting.

A bacterial display library produced in accordance with the present invention can be separated by affinity chromatography just as with the phage. Because bacterial cells are larger, care must be taken during 20 loading to prevent plugging and the non-specific retention of bacteria in the column. Subsequently, the cells can be eluted either by passing free antigen through the column or by low pH. Even though gram-negative bacteria are not as resistant to low pH as are phage, there is no meaningful decrease in cell vitality for at least 10 minutes at pH 3.3 [Martineul, *et al.*, 25 *Bio/Technology*, 9:170 (1991)]. Thus, elution by low pH and rapid neutralization can be employed for the isolation of strong binding clones.

The host cells displaying the desired target protein (display bacteria) may then be further cultured and used to obtain the fusion protein. If

desired, the target protein may be separated from the pilin protein and further purified using pilin purification techniques familiar to the artisan [*J. Bacteriology*, 146(1):251-259 (1981)].

- 5       Once a desired target protein has been displayed, one can mutate the DNA encoding the heterologous polypeptide, e.g., by use of a mutator strain, and use affinity separation technology to identify and select peptides that bind to one or more targets.
- 10      The display method of the present invention can be used for the detection and characterization of recombinant proteins. For example, the method can be used to map an uncharacterized epitope as follows: Sequences encoding either a library of (1) random peptides or (2) peptides derived from the immunoreactive protein of interest can be cloned into a
- 15      *traA* expression vector of the present invention, e.g., pPR35. *E. coli* host cells capable of forming F pili (e.g. XL1B) are then transformed with the vector bank and the peptide library-*traA* fusion proteins are displayed on the bacterial cell surface. Following growth on a solid substrate, e.g., a nylon membrane, the resulting bacteria are screened for expression of the
- 20      fusion protein that react with labeled antibody. Reactive colonies can then be picked and the vectors isolated. Sequence analysis of the DNA insert would reveal which of the cloned peptides sequences corresponded to the epitopes recognized by the antibody.
- 25      The display method of the present invention can also be for detecting recombinant protein activity e.g., antibodies. For example, the method can readily be applied to screening libraries of recombinant antibody-*traA* fusion proteins. These libraries may include combinatorial single-chain gene banks of heavy and light variable region genes or

- mutational libraries of specific recombinant antibody genes [Reviewed in Whitlow, M & Filpula, D. (1991). Methods: A Companion to Methods in Enzymology 2:97-105.] On the basis of the results set forth in Examples 6 and 7 indicating that the  $\alpha$ -CKMB scFv-traA fusion protein is folded into a 5 biologically active conformation, this method has general application to detection of recombinant protein activities displayed on the surface of the bacterial cell colony. The activities to be detected could include binding activities, catalytic activities, inhibitory activities and altered structural conformations.
- 10 The present invention can also be used as a primary cloning system. For example, a cDNA library can be constructed and inserted in a vector of the present invention and the library screened for the ability to bind a ligand. The ligand/binding molecule combination could include any pair of 15 molecules with an ability to specifically bind to one another, e.g., receptor/ligand, enzyme/substrate (or analog), nucleic acid binding protein/nucleic acid, etc. If one member of the complementary pair is available, this may be a preferred way of isolating a clone for the other of the pair.
- 20 As discussed above, it will often be necessary to increase the diversity of a population of genes cloned for the display of their proteins on a bacterial surface or to mutate individual nucleotide sequence. *In vitro* or *in vivo* mutagenesis techniques can be used for either purpose and are well 25 known to the skilled artisan. Alternatively, mutator strains can be used. A mutator strain is a strain which contains a genetic defect which causes DNA replicated with in it to be mutated with respect to its parent DNA. Such strains include those carrying the *mut* D5 mutation such as ES 1578. Therefore, if a population of genes is introduced into these strains, it will

be further diversified and can be transferred to a non-mutator strain if desired, for display and selection.

Since the F pilus acts a receptor for the RNA bacteriophage and 5 filamentous DNA phage, the display method of the present invention can make use of a binding protein on the phage to target the phage genome to a particular bacterial cell displaying a protein recognized by the phage. For example, instead of having the pilus/bacteriophage interaction that allows the phage to enter the cell, an antigen/antibody interaction can be used to 10 allow the bacteriophage to interact with the pili and then enter the cell. For filamentous phage, the product of gene III acts as the attachment protein, it is believed, through interactions with residues near the N-terminus of the pilin protein. The gene III protein is made up of specific domains involved in incorporation into the page coat, phage morphology, 15 interactions with the bacteria pilus, and entry into the bacteria cells, as depicted in Figure 10.

In addition to filamentous bacteriophage, RNA bacteriophage, such as Q $\beta$ , MS2, f2 and R17, specifically interact with the F pilus and infect 20 the cells. The ability to absorb to the pilus is conferred by maturation A protein (or A<sub>2</sub> for Q $\beta$ ) which is present in one copy per virion [Paranchysh, W. (1975) in RNA Phages, ed. N.D.Zinder. (Cold Spring Harbor Laboratory:New York). pp.85-112]. Like the gene III protein of filamentous bacteriophage, the RNA phage maturation A protein can be used to form 25 fusions without affecting infectivity.

In accordance with this method, a display bacteria is formed in which one protein of the specific binding pair is displayed and replaces the natural receptor for bacteriophage infection. A bacteriophage is also

altered such that the normal pilin interaction domain is substituted with the other member of the specific binding pair. This is accomplished by removing the region of the phage attachment protein (e.g. gene III protein of filamentous phage or the A protein of RNA phage) that encodes the pilin

5 binding domain and inserting in its place DNA that encodes the second member of the specific binding pair. The chimeric gene may be incorporated into the phage genome or a recombinant phagemid expression vector. The gene is then expressed in the appropriate strain, e.g. *E. coli*, and the fusion protein and the corresponding phage (or phagemid) genome

10 are packaged into the bacteriophage particles. The phage is then contacted with the display bacteria under standard conditions. Phage displaying one member of the specific binding pair recognize and infect the bacteria displaying the other member based on the protein-protein interactions between the displayed proteins. The phage genome is then

15 internalized by the display bacteria. Display bacteria infected with the phage genome can then be selected by, for example, identifying of a marker gene i.e., antibiotic resistance, transferred from the phage to the display bacteria. DNA encoding members of the specific binding pair can then isolated from the display bacterial host.

20 Using fd phage, for example, the phage is altered such that the normal pilin interaction domain (e.g., amino acid 107 to 197 of the gene III protein) is removed and replaced by a polypeptide which will specifically bind the target protein displayed on the display bacteria. Thus, the display phage recognizes and infects the display bacteria solely based on the

25 protein-protein interactions between the displayed recombinant proteins. Figure 11 shows the general characteristics of this system. The phage or phagemid genome is then internalized and expressed. Control signals for transcription, translation and replication can be present. It is particularly useful if the phage or phagemid genome contain sequences useful in

selecting for the desired target cell. Useful sequences include, for example, those conferring antibiotic resistance to the target cell.

Bacteriophage useful in the method of the present invention include  
5 filamentous phage and RNA phage that utilize as a receptor the pilin protein. Such phage include MS2, Q $\beta$ , M13, f1, fd and fd-tet. In addition, phagemid expression vectors derived from such filamentous phage can also be used. These vectors can carry plasmid and phage origins of replication and genes that confer antibiotic resistance. The preferred phage is fd-tet  
10 [Zacher, A.N., Stock, C.A., Golden, J.W. and Smith, G.P. (1980) Gene 9,127-140] and the preferred phagemid is a derivative of f1 phage such as pBC (Stratagene).

As an example of this method, the EE tag antigen is displayed on the  
15 bacteria pili as a traA fusion using a phagemid expression vector that has been developed to allow for recombinant proteins to be displayed on the surface of bacteriophage. Using this system, the anti-EE tag scFv is displayed on the surface of the bacteriophage particles as a fusion with the gene III protein that has the pilin binding region (amino acid 107-197)  
20 deleted (this protein will be referred to as genelllp ABS as shown in Figure 10). Interactions between the anti-EE tag scFv antibody and the EE tag antigen are measured by the ability of the display phage to infect the display bacteria. Specific strategies for generating the display bacteriophage and for measuring infection are set out in Example 8 below.  
25

Other recombinant proteins can be displayed on the bacteriophage and bacterial cell surface. These can include libraries of scFv genes displayed on the phage and a specific antigen peptide on the display bacteria. Screening for specific scFv-antigen interactions involves 1)

rescue of the scFv display phagemid particles and 2) mixing the phage with the antigen displaying bacteria and testing for the presence of a marker e.g., infectivity by growth on agar plates containing antibiotics (chloramphenicol). The method of the present invention does not require 5 antigen purification or the multiple rounds of enrichment and phage amplification steps that are currently required in phage display systems.

Phage or phagemid DNA would be isolated from the resulting antibiotic resistant colonies and the candidate scFv genes could be 10 sequenced. Once the initial characterization is completed, the candidate scFv genes could be subcloned into bacteria expression vectors for the production and further characterization of the single-chain antibodies.

A bacteriophage vector based system can also be constructed for 15 display of the recombinant proteins. Such a method has the advantages that it can be used to genetically select for high affinity protein-protein interactions and for binding affinity improvement when coupled with random or site-directed mutagenesis of the recombinant protein. As an example, an expression vector is constructed from the fd-tet phage by 20 replacing the normal genelll with the anti-EE tag scFv-genelllp ABS fusion gene as outlined in Figure 13. DH5- $\alpha$ F' cells are transformed to tetracycline resistance with the phage expression vector. The transformed cells are grown overnight, for example, in 100 ml of 2xYT media containing 15  $\mu$ g/ml tetracycline. The cells are removed by centrifugation 25 and the phage particles in the culture media can be concentrated by precipitation with, for example, 5% PEG and 0.5 M NaCl. The resulting phage particles carry the genelllp ABS phage vector and display the anti-EE tag scFv-genelllp ABS fusion protein of the bacteriophage surface. These phage particles are used to infect DH5- $\alpha$ F' cells carrying the EE tag-traA

fusion vector. Infectivity can be tested by selection of tetracycline resistant colonies on agar plates as previously described. Alternatively, since the expression phage is able to replicate and re-infect bacteria displaying the EE tag, infectivity can be characterized by the formation of 5 plaques on a lawn of the display bacteria or the propagation of the phage in liquid cultures of the display bacteria. Plaque size or phage titer liquid media provides an indication of the strength of the recombinant protein-protein interactions responsible for the phage infectivity and propagation. In other words, the highest affinity recombinant protein-protein interactions 10 between the display phage and the display bacteria results the highest infectivity rates. The specificity of the infection can be tested with cells that do not display the EE tag antigen.

This system is useful in screening libraries of recombinant protein 15 such as scFv. Phage displaying the high affinity scFv can infect and replicate in the antigen displaying bacteria at a higher rates than the phage displaying low affinity scFv. Thus, the phage displaying the high affinity scFv will be selectively enriched with continued growth of the culture. This is true for other specific binding pairs as well. The resulting phage DNA 20 can be isolated and the candidate scFv genes and proteins further characterized by sequence and affinity analyses.

This system can further be used to screen compounds, i.e., inhibitors or co-factors, that affect specific binding pair interaction. In this 25 screening method, the display bacteria and the display phage are mixed and infectivity of the display phage or phagemid particles is measured as previously described. One such detection method would be antibiotic-resistant growth of the display bacteria following infection with the display phage carrying the antibiotic resistance gene. Candidate compounds are

added to the binding reaction and the effect on the level of phage infectivity is measured. For example, the suppression of growth of the display bacteria in appropriate selective media is one means of screening a large number of candidate inhibitor molecules. Compounds potentiating 5 binding can be selected by screening for increased growth.

The present invention is further illustrated by the following Examples. These Examples are provided to aid in the understanding of the invention and are not construed as a limitation thereof.

10

The references cited above and below are herein incorporated by reference.

#### EXAMPLE 1

15

#### **CONSTRUCTION OF A *traA* FUSION VECTOR FOR EXPRESSING PROTEINS ON THE BACTERIAL SURFACE**

A system was designed to allow inducible expression and display of polypeptides fused to the amino terminus of the pilin protein on the surface 20 of bacteria. In this system, the gene encoding the polypeptide of interest was cloned into the *traA* vector, pPR35 and expressed in an *F<sup>+</sup>* bacteria strain. The *traA* expression vector is based on the multicopy pUC19 vector with features shown in Figure 1. The *traA* leader and *traA* protein (pilin) DNA fragments were cloned downstream of the inducible *lacZ* 25 promoter of pUC19. The *traA* leader allows for proper processing and display of the pilin fusion protein. Cloning sites for *Ncol*, *Sfi* and *Not* I were incorporated between the *traA* leader and pilin polypeptide sequences to allow insertion of foreign DNA sequences. In addition, a DNA sequence encoding the EE tag antigen was cloned between the *traA* leader and *traA*

protein sequences to allow for detection of the fusion protein and characterization of the expression-display system.

The steps required to construct the pPR35 vector are outlined in Figure 2 and detailed as follows. The *traA* leader and *traA* protein gene fragments were amplified separately by PCR from an F plasmid template. The primers used in the amplification are described in Figure 2 and Figure 3. Typical PCR amplification reactions (100 µl) contained 10<sup>5</sup> boiled XL1B bacteria cells carrying the F plasmid as source of template DNA, 10 pmoles of the appropriate primers, 2.5 units of *Taq* polymerase, 100µM dNTP, 50mM KCl, 10mM Tris-HCl, pH 8.3, 1.5mM MgCl<sub>2</sub>, 0.01% gelatin. The template was denatured by an initial incubation at 96°C for 5 min. during which the *Taq* polymerase was added to hot-start the reaction. The desired products were amplified by 10 thermal cycles of 55°C for 1 min., 70°C for 1 min. and 96°C for 1 min. followed by 20-step cycles of 70°C for 1 min. and 96°C for 1 min. Amplification with the primers results in the addition of an *EcoRI* site on the 5' end of *Ncol* and *BamHI* sites on the 3' end of the *traA* leader fragment and *BamHI* and *Kasl* sites on the 5' end and an *XbaI* site on the 3' end of the *traA* protein fragment. The PCR products from 5 reactions were pooled, precipitated with 2 volumes of ethanol/0.3M sodium acetate, and the resulting products (about 0.2 µg of DNA) were resuspended in water. The *traA* leader PCR product was digested with *EcoRI* and *BamHI* and the *traA* protein PCR fragment was digested with *BamHI* and *XbaI*. The digested fragments were resolved by agarose gel electrophoresis and purified by elution from the agarose gel. In order to clone these fragments, a vector referred to as pPR5 was generated by digesting pUS18 DNA with *Kasl*, filling-in the site with Klenow DNA polymerase and religating the blunt ends. The purified digested PCR products were then ligated into *EcoRI/XbaI* digested pPR5.

Bacteria transformed with this ligation mix were screened for the product on the three fragment ligation. Shown in Figure 2, this vector is referred to as pPR2. Finally, the EE tag linker sequence was generated by two complementary oligonucleotide which was annealed have a *Ncol* sticky end 5 at the 5' end and a *KasI* sticky end at the 3' end. The annealed oligonucleotide were ligated into *Ncol/KasI* digested pPR2 to give the *traA* fusion vector, pPR35. The sequence of pPR35 is shown in Figure 4.

## EXAMPLE 2

10

### ISOLATION OF SINGLE-CHANGE ANTIBODY GENE AND CLONING INTO THE *traA* FUSION VECTOR

The *traA* fusion vector has been designed to express both peptide 15 antigens such as the EE antigen as well as other recombinant proteins such as single chain antibodies. For the purpose of this example, single-chain antibody genes were created in which the heavy and light variable regions of a particular monoclonal antibody were joined together by a flexible polypeptides linker. Single-chain antibody (scFv) genes were generated 20 from a monoclonal antibody (TA1) directed against the prothrombin polypeptide F1.2 and from a monoclonal antibody directed against creatine kinase-MB ( $\alpha$ -CKMB) as described below and outlined in Figure 5. For the TA1-ScFv, the first step involved poly-A RNA isolation from TA1 hybridoma cells by using the Fast-track RNA isolation kit (Invitrogen) 25 according to manufacturer's procedures. This RNA (1/10 of the mRNA isolated was used) was converted to cDNA using Superscript-MLV Reverse Transcriptase (GIBCO-BRL) and oligo-dT specific priming according to manufacturer's procedures. Of the 20  $\mu$ l of cDNA generated, 2  $\mu$ l was used as template DNA for PCR. The PCR primers for amplifying the TA1 30 mAb heavy and light chain variable region genes are JS135/JS134 and

JS133/JS153, respectively, as shown in Figure 2. The PCR buffer conditions are the same as described in Example 1. The template was denatured by an initial incubation at 96°C for 5 min. during which the *Taq* polymerase was added to hot-start the reaction. The immunoglobulin 5 variable region gene fragments were amplified by 10 thermal cycles of 48°C for 1 min., 70°C for 1 min., and 96°C for 1 min. followed by 25-step cycles of 70°C for 1 min. and 96°C for 1 min. The desired products (about 260 bp) were resolved by agarose gel electrophoresis and purified by elution from the agarose gel. These fragments were then used as DNA 10 templates in PCRs to attach a 45 nucleotide linker sequence to the 3' end of the heavy chain and the 5' end of the light chain variable gene fragment, resulting in the addition of a flexible 15 amino acid peptide linker to the variable region polypeptides. The PCR primers used in the linker attachment are JS135/JS139 and JS137/JS153 for the heavy and light 15 chain variable gene fragments, respectively. The PCR conditions were 10 thermal cycles of 48°C for 1 min., 70°C for 1 min., and 96°C for 1 min., followed by 25-step cycles of 70°C for 1 min., and 96°C for 1 min. Following resolution by agarose gel electrophoresis, the desired products (about 400 bp) purified by elution from the agarose gel. Sequence-overlap 20 extension PCR was used to link the heavy and light chain variable gene fragments by first annealing and extending the heavy chain + light chain variable + linker gene fragments for 10 thermal cycles of 52°C for 1 min., 70°C for 1 min., and 96°C for 1 min. The linked fragments were then amplified by the addition of JS135/JS153 primers and 15 additional step 25 cycles of 70°C for 1 min. and 96°C for 1 min. The desired products (about 720 bp) were purified as described above. Initially, the TA1 scFv gene fragment was digested with *Ncol* and *Spel* and ligated into the pJS102 cloning vector digested with *Ncol/Spel*. The resulting construct was sequenced to verify that it contains the TA1 scFv gene. The

pJS102/TA1 scFv plasmid was then used as template DNA to PCR the TA1 scFv gene fragment in order to add a *NotI* site to the 3' end of the light chain variable gene. The primers used were JS135/JS153 and the PCR conditions were 10 thermal cycles of 48°C for 1 min., 70°C for 1 min. and 96°C for 1 min. followed by 25-step cycles of 70°C for 1 min. and 96°C for 1 min. The desired products (about 720 bp) were resolved by agarose gel electrophoresis and purified by elution from agarose gel. The TA1 scFv gene fragments were digested with *Ncol* and *NotI* and ligated into the pPR35 *traA* expression vector digested with *Ncol/NotI*, resulting the creation of the TA1 scFv/EE tag/*traA* fusion vector, pGH21.

The same strategy was used to isolate the variable region genes from Conan  $\alpha$ -CKMB hybridoma cell line and to construct the  $\alpha$ -CKMB scFv gene. The corresponding heavy and light chain PCR primers are shown in Figure 3. Following the sequence-overlap expression PCR step, the  $\alpha$ -CKMB scFv gene fragment was digested *Ncol* and *Spel* and ligated into the pGH21 *traA* expression vector digested with *Ncol/Spel*, essentially swapping the TA1 scFv gene for the  $\alpha$ -CKMB scFv gene. The resulting construct is referred to as  $\alpha$ -CKMB scFv-*traA*.

20

### EXAMPLE 3

#### PRODUCTION OF *traA* FUSION PROTEINS

25 The *traA* expression system was characterized in several ways. First, bacterial expression of the TA1 scFv-EE tag-*traA* or  $\alpha$ CKMB scFv-EE tag-*traA* fusion protein was examined by immunoblot analysis. The pGH21 and  $\alpha$ -CKMB scFv-*traA* vectors were transformed into XL1B cells carrying the F plasmid. Correct candidates were screened by restriction analysis of

alkaline-SDS miniprep DNA and verified by DNA sequencing. To induce the expression of the *traA* fusion protein, 60  $\mu$ l of an overnight culture was used to inoculate 3 ml of 2xLB media, 50  $\mu$ g/ml ampicillin, 15  $\mu$ g/ml tetracycline. Following a 2 hour incubation at 37°C, isopropyl-1-thio  $\beta$ -D-galactoside (IPTG) was added to 2mM final concentration. After 4 hours at 37°C, the OD<sub>600</sub> of the culture was determined and 2 ml of the culture was harvested by microcentrifugation for 5 min. The cell pellet was frozen at -70°C and then was resuspended at 10 ODs/ml in cold TxTBS (0.1% Triton C-100, 10mM Tris-HCl, pH 7.4, 0.15M NaCl). The cells were sonicated for 3 to 5 min. and the cell debris removed by microcentrifugation at 10,000 x g for 10 min. at 4°C. The supernatant (10  $\mu$ l) was mixed with SDS/ $\beta$ -mercaptoethanol loading buffer and boiled for 5 min. to denature the proteins. The samples were resolved by SDS-polyacrylamide gel electrophoresis on 12.5% polyacrylamide gels. The material in the gels was transferred to PVDF nylon membranes using a semi-dry transblot apparatus. The membrane was blocked overnight at 4°C with 20 ml of blocking buffer (0.5% NP-40, 0.5% non-fat dried milk in PBS) and probed with 20 ng/ml anti-EE tag mAb conjugated to horseradish peroxidase (anti-EE tag mAb-HRP). The anti-EE tag mAb-HRP was detected by the ECL reagent (Amersham). The signal for the  $\alpha$ -CKMB scFv-*traA* fusion protein was detected at the expected molecular weight of 40 kD, while lysates from XL1B/vector alone showed no signal. The TA1 scFv-*traA* fusion protein migrates at 46 kD, however, the TA1 scFv protein migrates through SDS-PA gels at a higher molecular weight than expected. The TA1 scFv-*traA* fusion protein was also detected in the growth media, consistent with the fact that F pili can detach from the cell surface and be found in the media.

- 30 -

XL1-B/pGH21 cells (1L) were grown and TA-1/EE tag/traA fusion protein expression was induced with IPTG as described in Example 2. The bacteria pilin protein was partially purified by shearing the pili from the cells and PEG precipitation as described by Moore, et al. [J. Bacteriology, 5 146(1):251-259 (1981)]. The fusion proteins present in the induced cells (cell lysate lane) and in the partially purified protein (PEG ppt lane) were examined by Western analysis using the anti-EE tag mAb-HRP as a probe. See, Figure 15. The band corresponding to the TA-1/EE tag/traA fusion protein is indicated. The immunoreactive material at the top of the 10 stacking gel is aggregated fusion protein that does not enter the resolving gel.

#### EXAMPLE 4

15 **DETECTION OF THE ANTIGEN-traA FUSION PROTEIN  
ON THE BACTERIAL SURFACE BY CLONING SCREENING**

The traA expression system was used to develop improved methods for the detection of recombinant proteins. Two simple detection methods 20 were performed to test whether the antigen-traA fusion protein was displayed on the surface of the bacteria cells. The first was an immunodetection method for screening for bacterial colonies grown on nylon membranes. The XL1B strain expressing the TA1-EE tag-traA fusion protein was spread on a nylon membrane and the membrane was placed 25 on 2xLB agar plate containing 50 µg/ml ampicillin and 15 µg/ml tetracycline for selection of the vector and XL1B strain, respectively. For induction of the traA fusion gene expression, the membrane was prewet with 10mM IPTG. Following overnight incubation at 37°C, the membrane was removed from plate and washed 3 times by cold Imidazole buffer saline 30 (IBS - 40 mM Imidazole, pH 7.0, 0.15M NaCl). Membrane was blocked

with 0.5% milk-PBS with agitation at 4°C for 1 hour, and then incubated for 1 to 2 hours with 43 ng/ml anti-EE tag mAb-HRP in IBS at 4°C. Following 5 washes with IBS at 4°C, the membranes were reacted with ECL reagents and the immunoreactive material was detected. By this colony immunoblot methodology, anti-EE tag mAb-HRP recognized the IPTG-induced XL1B/TA1-EE tag-traA colony but not the non-induced XL1B/TA1-EE tag-traA colony. XL1B cells carrying a control vector (no EE tag-traA) failed to give any signal. The specificity of binding of anti-EE tag mAb on cell surface was also determined by incubating the colony membrane with an antibody to a different peptide tag (KT3). No signal was detected on these membranes.

The bacteria colony immunodetection method was also applied to epitope mapping analysis. To test this method, mixtures of XL1B cells carrying either the TA1-EE tag-traA or the control vector (no EE tag-traA insert) were grown overnight on 2xLB agar plates containing 50 µg/ml ampicillin and 15 µg/ml tetracycline overnight. The colonies were replica-plated onto nylon membranes and placed on 2xLB agar plates containing 10mM IPTG, 50 µg/ml ampicillin and 15 µg/ml tetracycline. Following growth at 37°C, the colonies on the membranes were probed with anti-EE tag mAb-HRP as described above. In the IPTG induced samples, positive signals were detected for single colonies as shown in Figure 6. The corresponding colonies were picked from the master plate for characterization and were found to carrying the TA1-EE tag-traA vector.

25

#### EXAMPLE 5

#### WHOLE CELL ELISA TO DETECT ANTIGEN EXPRESSED ON THE BACTERIAL SURFACE

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The second method to test the accessibility of the antigen-traA fusion protein on the surface of the bacteria was an ELISA method with intact cells. Cells grown to early log-phase were induced by IPTG for 4 hours at 37°C. The cells were harvested and resuspended in cold PBS to 5 1.0 OD<sub>595</sub>/ml. This step will remove any traA fusion protein present in the media that is not associated with the cells. Microtiter plates were coated with 100µl of bacterial dilution per well. After overnight incubation at 4°C, unattached cells were discarded and wells were blocked with PBS containing 1% bovine serum albumin for 1 hour at 4°C. Following the 10 blocking step, the wells were incubated with 100 µl of 0.34 µg/ml anti-EE tag antibody HRP. After 5 times washes with PBS, antigen-antibody complexes were developed by HRP-ELISA substrate (H<sub>2</sub>O<sub>2</sub>, ABTS peroxidase substrate). Reaction values were recorded by ELISA reader. The OD<sub>450</sub> reading indicates the amount of anti-EE tag mAB-HRP activity 15 captured in each well and correlates with the amount of EE tag fusion protein expressed on the cell surface. The induced XL1B/TA1-EE tag-traA samples showed greater than six-fold higher readings than the non-induced sample or the XL1B/control vector (no EE tag-traA insert) sample as shown in Figure 7, indicating that this method is applicable to specifically 20 detecting antigens presented on the cell surface. By adding known amounts of peptide antigen and antibody to the binding reaction, this method could be used to quantitative antibody/antigen binding. In addition, the epitope could be characterized in a comparative ELISA assay format where the effect of different peptides on antibody/antigen-traA 25 fusion protein interaction is determined.

#### EXAMPLE 6

30 **WHOLE CELL ELISA TO DETECT THE ACTIVITY OF  
A RECOMBINANT ANTIBODY DISPLAYED ON THE BACTERIAL SURFACE**

The results from Examples 4 and 5 indicate that antigens fused to the traA protein could be displayed on the surface of bacteria and could be specifically detected by the corresponding antibody. Similar experiments were carried out to determine if a functional recombinant protein could be displayed of the bacterial surface as described below. To detect the activity of recombinant single chain anti-CKMB Ab displayed on the bacteria cell surface, an ELISA method with intact cells was performed. Cells carrying either the  $\alpha$ -CKMB scFv-EE tag-traA fusion vector or the control vector (TA1-EE tag-traA fusion vector or a vector without an insert) were grown to early log-phase at 37°C. At that point, expression of the fusion protein was induced by the addition of 0.2mM IPTG for 4 hours at 37°C. The cells were harvested and resuspended in cold PBS to 10.0 OD<sub>595</sub>/ml. Microtiter plates were coated with 100  $\mu$ g/ml anti-CK-BB mAb in coating buffer (0.1M Tris-HCl, pH8.5) and were incubated overnight at 4°C. Unattached anti-CK-BB mAb was discarded and the wells were washed once with washing buffer (0.1 M Tris-HCl, pH 7.4, 1.0M NaCl, 0.1% NaN<sub>3</sub>). The wells were incubated with 100 $\mu$ l of 0.3  $\mu$ g/ml CK-MB in dilution buffer (2% gelatin, 0.1% Tween 20 in 0.01m Tris-HCl, pH7.3, 0.15 M NaCl) at room temperature for 1 hour with agitation. The wells were washed once with rinse buffer (0.01M Tris-HCl, pH7.3, 0.15M NaCl, 0.2% BSA, 0.05% Tween-20, 0.2% NaN<sub>3</sub>). 100  $\mu$ l of cell suspension was added to each well and the plate was incubated at room temperature for 1 hour with agitation. The unattached cells were discarded and the wells were washed twice with rinse buffer. The wells were incubated with 100 $\mu$ l of 0.34 $\mu$ g/ml anti-EE tag mAb-HRP conjugate in dilution buffer at room temperature for 1 hour with agitation. After washing the wells twice with rinse buffer, the HRP-ELIA substrate (H<sub>2</sub>O<sub>2</sub>, ABTS peroxidase substrate) was added and developed for 20 min. The amount of color development as determined by an ELISA reader at 450 nm corresponds to

the amount of scFv-EE tag-traA fusion protein detected in the well. These values correlate with the amount of anti-CKMB scFv activity displayed of the cell surface that is captured by the immobilized CK-MB. Typical results are shown in Figure 8. The IPTG-induced XL1B/α-CKMB scFv-EE tag-traA 5 plasmid sample showed 29-fold higher levels of captured α-CKMB scFv-EE tag-traA fusion protein than the non-induced XL1B/TA1-EE tag-traA cells that express a different recombinant antibody which does not recognize CK-MB. The XL1B cells carrying the control vector did not express EE tag-traA protein and showed no activity in this assay.

10

The results of these experiments indicate that the α-CKMB scFv domain of the fusion protein is folded into a biologically active conformation and is displayed on the surface of the cells. The combination of the single-chain antibody and the EE antigen tag on the same display 15 protein allows for versatility in the development of ELISA formats. The sandwich capture ELISA format used in this Example is just one of the many possibilities.

#### EXAMPLE 7

20

##### DETECTION OF FUNCTIONAL SINGLE-CHAIN ACTIVITY OF THE BACTERIAL SURFACE BY COLONY SCREENING

This example demonstrates the successful detection of a recombinant protein activity expressed on the surface of the bacterial cell 25 colony. A mixture of the XL1B strain carrying the α-CKMB scFv-EE tag-traA fusion vector and the strain carrying the TA1-EE tag-traA fusion vector was distributed evenly on a nylon membrane. The membrane was placed on a 2xLB agar plate containing 50μg/ml ampicillin and 15μg/ml tetracycline and incubated at 37°C until small bacterial colonies appeared. 30 At this time, a replica membrane was made by overlaying the master

membrane with a new membrane. The replica membrane was then removed and cut in half. One half was incubated on a 2xLB agar plate containing 10mM IPTG, 50 $\mu$ g/ml ampicillin and 15 $\mu$ g/ml tetracycline and the other half was on a 2xLB agar plate containing just 50 $\mu$ g/ml ampicillin  
5 and 15 $\mu$ g/ml tetracycline. Following the overnight incubation at 37°C, the IPTG-induced and non-induced membranes were removed and washed 3 times by IBS. Membranes were blocked with 20ml of 0.5% milk-IBS with agitation at 4°C for 1 hour, and then incubated for 1 hour at 4°C with 20 ml of 0.3  $\mu$ g/ml CKMB in the dilution buffer. Following 3 washes with cold  
10 IBS, the membranes were blocked at 4°C for 1 hour, and with anti-CK-BB mAb conjugated to alkaline phosphatase. Following 5 washes with cold IBS, the membranes were reacted with Lumiphos 53 (Boehringer Mannheim). The immunoreactive material was detected by fluorography and the typical results are shown in Figure 9. Strong positive signals  
15 corresponding to single colonies were detected in the IPTG-induced samples. These colonies were picked from the master plate and found to carry the  $\alpha$ -CKMB scFv-EE tag-traA vector. The colonies corresponding to negative signal on the film contained the control vector. The results indicate that the  $\alpha$ -CKMB scFv-traA fusion protein is displayed of the  
20 bacterial surface and the single-chain antibody is folded into a biologically active conformation.

This method provides an easy rapid procedure for detecting recombinant single-chain antibody activity. It could be readily applied to screening libraries of recombinant antibody-traA fusion proteins. These libraries may include combinatorial single-chain gene banks of heavy and light variable region genes or mutational libraries of specific recombinant antibody genes. On the basis of the results indicating that the  $\alpha$ -CKMB scFv-traA fusion protein is folded into a biologically active conformation,  
25

this method could have general application to detection of recombinant protein activities expressed on the surface of the bacterial cell colony. The activities to be detected could include binding activities, catalytic activities, inhibitory activities and altered structural conformations.

5

### EXAMPLE 8

#### BACTERIOPHAGE/PILIN INTERACTION SYSTEM

A phagemid vector was designed for the expression and display of genelllp ΔBS fusion proteins on the surface of the bacteriophage particle. The phagemid vector is based on the pBC phagemid vector (Stratagene) with features shown in Figure 12. This vector carries the ColE1 replication origin for plasmid propagation, the f1 filamentous phage replication origin for recovery of phagemid DNA following co-infection with helper phage and the chloramphenicol resistance gene for antibiotic selection. The *pe/B* leader and genelllp ΔBS DNA fragments were cloned downstream of the inducible *lacZ* promoter of pBC. The *pe/B* leader was designed to allow for proper processing and display of the fusion protein on the bacteriophage particle. Cloning sites for *Ncol*, *Sfil*, *Spel*, and *Notl* were incorporated between the *pe/B* leader and genelllp ΔBS sequences to allow insertion of foreign DNA sequences. The steps involved in constructing this vector (referred to as LE2) are shown in Figure 12.

The anti-EE tag scFv gene are isolated from monoclonal hybridoma mRNA as outlined in Example 2 and are inserted at the *Notl* and *Spel* sites of LE2. An F' host strain, DH5-*αF'* [Woodcock, D:M. et al (1989) Nucl.Acids. Res. 17,3469-3478] is used to propagate these vectors by growth in media containing 30 µg/ml chloramphenicol.

In order to rescue phagemid particles, DH5- $\alpha$ F' cells carrying phagemid expression vector are transformed with the fKN16 phage DNA to tetracycline resistance. The fKN16 phage derivative was constructed from the tetracycline-resistance phage, fd-tet, by deleting a 507 bp segment of gene III (Nelson, et al., *Virology*, 108:338-350 (1981)). This phage is non-infective due to the gene III deletion but provides the helper phage proteins necessary for replication and packaging of the phagemid expression vector. DH5- $\alpha$ F' cells carrying both the phagemid expression vector and fKN16 are grown overnight in 100 ml of 2xYT media containing 30  $\mu$ g/ml chloramphenicol and 15  $\mu$ g/ml tetracycline. The cells are removed by centrifugation and the phage particles in the culture media are concentrated by precipitation with 5% PEG and 0.5 M NaCl. The resulting phage particles carry either the fKN16 phage (tet') or the genelllp ABS phagemid vector (chl'). Both the defective fKN16 gene protein and the anti-EE tag scFv-genelllp ABS fusion protein are displayed on the bacteriophage surface. The rescued phage are used to infect XL-1B cells carrying the EE tag-traA fusion vector. Since these cell express the EE tag antigen on their pili, the bacteriophage displaying the anti-EE tag scFv-genelllp ABS fusion protein bind the EE tag-traA fusion protein and infect these cells resulting in chloramphenicol resistant clones carrying the phagemid expression vector.

A bacteriophage vector based system can also be constructed for the display of recombinant proteins. The expression vector can be constructed from the fd-tet phage by replacing the normal genelll with the anti-EE tag scFv-genelllp ABS fusion gene as outlined in figure 14. DH5- $\alpha$ F' cells are transformed to tetracycline resistance with the phage expression vector. The transformed cells are grown overnight in 100 ml of 2xYT media containing 15  $\mu$ g/ml tetracycline. The cells will be removed by

- centrifugation and the phage particles in the culture media will be concentrated by precipitation with 5% PEG and 0.5 M NaCl. The resulting phage particles will carry the genelllp ΔBS phage vector and display the anti-EE tag scFv-genelllp ΔBS fusion protein of the bacteriophage surface.
- 5 These phage particles will be used to infect DH5- $\alpha$  F' cells carrying the EE tag-traA fusion vector.

Infectivity can then be tested by selection of tetracycline resistant colonies on agar plates as described for the phagemid system.

10 Alternatively, since the expression phage is able to replicate and re-infect bacteria displaying the EE tag, infectivity can be characterized by the formation of plaques on a lawn of the display bacteria or the propagation of the phage in liquid cultures of the display bacteria. Plaque size or phage titer in the liquid media provide an indication of the strength of the

15 recombinant protein-protein interactions responsible for the phage infectivity and propagation. In other words, the highest affinity recombinant protein-protein interactions between the display phage and the display bacteria provide the highest infectivity rates. The specificity of the infection can also be tested with cells that do not display the EE tag

20 antigen.

This system is useful in screening libraries of recombinant protein such as scFvs. The phage displaying the high affinity scFv will infect and replicate in the antigen displaying bacteria at a higher rate than the phage

25 displaying low affinity scFv. Thus, the phage displaying the high affinity scFv will be selectively enriched with continued growth of the culture. The resulting phage DNA can be isolated and the candidate scFv genes and proteins could be further characterized by sequence and affinity analyses.

It should be understood that the Examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled and purview of this Applications and the scope of the appended claims.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Huang, Grace P.  
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Stinson, Jeffrey R.  
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(ii) TITLE OF INVENTION: A METHOD FOR DISPLAYING  
PROTEINS

(iii) NUMBER OF SEQUENCES: 24

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## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

## (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/258026  
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(C) CLASSIFICATION:

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## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: unknown  
(D) TOPOLOGY: unknown

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGGGGGAATT CTATCCGAAA TTGAGGTAAC TTATG

35

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 39 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: unknown

(D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGGGGGTCTA GATTATCAGA GGCCAACGAC GGCCATAAC

39

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGGGGGATCC CCATGGCCAG CTGCGGGAAG AACATCATCA G

41

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGGGGGATCC GGCGCCGGCA GCAGTGGTCA GGACCTGATG

40

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CACTTGCCA TGGCCGAGGT TCAGCTGCAG CAG

33

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCTGCCACCG CCACCTGAGG AGACGGTGAC TGAG

34

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGAGGCCGGCG GTTCTGATAT TGTGATGACT CAGGC

35

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TTCATAGGCG GCCGCACTAG TAGCMCGTT CAGYTCCARC

40

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GCCGGCCATG GCCCAGGTBC ARCTKMARSA RTC

33

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GCTGCCACCG CCACCTGMRG AGACDGTGAS TGARG

35

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GGAGGCCGGCG GTTCTGACAT TGTGMTGWCA CAGTC

35

## (2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 40 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: unknown
  - (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TTCATAGGCG GCCGCACTAG TAGCMCGTTT KATYTCCARC

40

## (2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 45 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: unknown
  - (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGTGGCGGTG GCAGCGGCCGG TGGTGGTTCC GGAGGCAGCG GTTCT

45

## (2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 45 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: unknown
  - (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AGAACCGCCG CCTCCGGAAG GAGGACCGCC GCTGCCACCG CCACC

45

## (2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 53 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: unknown
  - (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CATGGCGGCC GGCAAGCGCGG CCGCTGAGGA AGAAGAGTAC ATGCCGATGG AAC

53

## (2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 53 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: unknown
  - (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GCGCCTTCCA TCGGCATGTA CTCTTCTTCC TCAGCGGCCG CGCTGCCGGC CGC

53

## (2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 540 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: unknown
  - (D) TOPOLOGY: unknown

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GAAACAGCTA TGACCATGAT TACGAATTCT ATCCGAAATT GAGGTAACCT ATGAATGCTG	60
TTTTAAGTGT TCAGGGTGCT TCTGCGCCCG TCAAAAAGAA GTCGTTTTT TCCAAATTCA	120
CTCGTCTGAA TATGCTTCGC CTGGCTCGCG CAGTGATCCC GGCTGCTGTT CTGATGATGT	180
TCTTCCCGCA GCTGGCCATG CGGGCCGGCA GCGCGGCCGC TGAGGAAGAA GAGTACATGC	240
CGATGGAAGG CGCCGGCAGC AGTGGTCAGG ACCTGATGGC AAGCGGTAAC ACCACGGTTA	300
AGGCGACCTT CGGTAAGGAC TCCAGTGTG TTAAATGGGT TGTTCTGGCT GAAGTTCTGG	360
TCGGTGCTGT CATGTACATG ATGACCAAAA ACCTCAAGTT CCTGGCCGGT TTTGCCATCA	420
TCTCTGTATT TATTGCTGTG GTTATGGCCG TCGTTGGCCT CTGATAATCT AGAGTCGACC	480
TGCAGGCATG CAAGCTTGGC ACTGGCCGTC GTTTACAAAC GTCGTGACTG GGAAAACCCT	540

## (2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 137 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: unknown
  - (D) TOPOLOGY: unknown

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Met Asn Ala Val Leu Ser Val Gln Gly Ala Ser Ala Pro Val Lys Lys	
1 5 10 15	
Lys Ser Phe Phe Ser Lys Phe Thr Arg Leu Asn Met Leu Arg Leu Ala	
20 25 30	
Arg Ala Val Ile Pro Ala Ala Val Leu Met Met Phe Phe Pro Gln Leu	
35 40 45	
Ala Met Ala Ala Gly Ser Ala Ala Glu Glu Glu Tyr Met Pro	
50 55 60	
Met Glu Gly Ala Gly Ser Ser Gly Gln Asp Leu Met Ala Ser Gly Asn	
65 70 75 80	
Thr Thr Val Lys Ala Thr Phe Gly Lys Asp Ser Ser Val Val Lys Trp	
85 90 95	
Val Val Leu Ala Glu Val Leu Val Gly Ala Val Met Tyr Met Met Thr	
100 105 110	
Lys Asn Val Lys Phe Leu Ala Gly Phe Ala Ile Ile Ser Val Phe Ile	

115	120	125
Ala Val Val Met Ala Val Val Gly Leu		
130	135	

## (2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 67 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: unknown
  - (D) TOPOLOGY: unknown

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GGGGGGGAGCT CTCTGCAAAG AGACAGTCAT AATGAAATAC CTATTGCCTA CGGCAGCCGC	60
TGGATTG	67

## (2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 66 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: unknown
  - (D) TOPOLOGY: unknown

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GGGGGGGCCGC GGCGCGGCC ATGGCCGGCT GGGCCGCGAG TAATAACAAT CCAGCGGCTG	60
CCGTAG	66

## (2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 84 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: unknown
  - (D) TOPOLOGY: unknown

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GGGGGGGCCGC GGCGCGGGAG GAAGAAGAGT ACAACCCGAA CGAAGGCGCC GCCTAGACTG	60
TTGAAAGTTG TTTAGCAAAA CCTC	84

## (2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 44 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: unknown
  - (D) TOPOLOGY: unknown

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GGGCCGAATT CCTATTAAGA CTCCTTATTA CGCAGTATGT TAGC	44
--	----

## (2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 53 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: unknown
  - (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GGGGGGACTA GTGCAGCCGC GGGCGCCGCT GAAACTGTTG AAAGTTGTTT AGC

53

## (2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 35 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: unknown
  - (D) TOPOLOGY: unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GGGGGGGGAT CCAGAGGGTT GATATAAGTA TAGCC

35

**WHAT IS CLAIMED IS:**

1. A method for displaying a target protein on a bacterial host cell comprising:

expressing a fusion protein in a bacterial host capable of forming a pilus, said fusion protein encoded by a chimeric DNA comprising a DNA segment encoding a leader amino acid sequence capable of mediating secretion of the fusion protein, a DNA segment encoding pilin subunits capable of forming said pilus, and a DNA segment encoding the target protein, said DNA segments being operably linked such that the host cell displays said target protein at its surface.

2. The method of claim 1, wherein said pilus is a receptor for bacteriophage attachment and infection.

3. The method of claim 2, wherein said pilus is an F pilus.

4. The method of claim 3, wherein the DNA segment encoding a leader segment encodes the traA leader sequence.

5. The method of claim 3, wherein the DNA segment encoding the pilin subunit encodes the tra A gene product.

6. The method of claim 1, wherein the DNA segment encoding the target protein is linked between the DNA segment encoding the leader and the 5' end of the DNA segment encoding the pilin subunit.

7. The method of claim 1, wherein the bacterial host cell is *E. coli*.

8. The method of claim 1, wherein the chimeric DNA further comprises an inducible promoter operably linked thereto.

9. The method of claim 1, wherein said chimeric DNA is carried within a recombinant vector.

10. A method for selecting a recombinant target protein from a DNA library including DNA encoding said recombinant target protein comprising:

(a) displaying the recombinant proteins encoded by said DNA library by the method of claim 1; and

(b) selecting bacterial host cells displaying the desired target recombinant protein.

11. The method of claim 1 or 10, wherein the bacterial host is a mutator strain which introduces genetic diversity into the target protein.

12. The method of claim 10, wherein the target protein is an antigenic determinant polypeptide.

13. The method of claim 10, wherein the bacterial host cells displaying the desired target recombinant protein is selected by immunoreactivity against an antibody.

14. A method for isolating a member of a specific binding pair comprising:

(a) expressing a fusion protein in a bacterial host capable of forming a pilus that is a receptor for bacteriophage attachment and infection, said fusion protein encoded by a chimeric DNA comprising a DNA segment encoding a leader amino acid sequence capable of mediating

secretion of the fusion protein, DNA segment encoding a pilin subunits capable of forming a pilus, and a DNA encoding said member;

(b) contacting the bacterial host of step (a) with a bacteriophage displaying an attachment protein having a pilin interaction domain wherein the DNA encoding the pilin interaction domain has been substituted with DNA encoding the other member of the specific binding pair; and

(c) selecting bacterial host cells recognized by the phage.

15. The method of claim 14, wherein said pilus is an F pilus.

16. The method of claim 15, wherein the phage is a filamentous phage or RNA bacteriophage.

17. The method of claim 16, wherein the phage is an fd phage.

18. The method of claim 14, wherein the DNA encoding the member is from a DNA library.

19. The method of claim 14, wherein the DNA encoding the other member is from a DNA library.

20. The method of claim 14, wherein the DNA encoding the member encodes an antigenic determinant and the DNA encoding the other member encodes an antibody.

21. The method of claim 14, wherein the DNA encoding the member encodes an antibody and the DNA encoding the other member encodes an antigenic determinant.

22. The method of claim 17, wherein the bacteriophage attachment protein is the gene III protein.

23. The method of claim 14, wherein the bacterial host recognized by the phage is selected by identification of a marker gene transferred from the phage to the host.

24. The method of claim 14, wherein the DNA encoding a member of the specific binding pair is mutagenized and specific binding pairs having an increased affinity are selected.

25. A method of screening for compounds affecting specific binding pair interaction comprising:

(a) contacting a bacterial cell having a specific binding pair-pilin fusion protein displayed on its surface with a bacteriophage altered such that the pilin interaction domain is substituted by the other member of the specific binding pair;

(b) adding a test compound; and

(c) measuring the effect of the test compound on the phage/bacterial cell interaction.

26. The method of claim 25, wherein the effect of the test compound on the phage bacterial cell interaction is measured by measuring the effect of the test compound on the infectivity of the display phage.

27. A method of screening for a member of a specific binding pair having increased binding affinity comprising:

(a) contacting a display bacteria displaying a pilin-specific binding member fusion with a bacteriophage altered such that the normal pilin

binding domain is substituted with a library of proteins containing the other member of the specific binding pair under conditions that allow phage infection; and

(b) identifying specific binding pairs having increased binding affinity by identifying bacteriophage having an increase frequency of infectivity.

28. A bacterial host cell displaying a target protein-pilin fusion.

29. The bacterial host cell of claim 28, wherein the host is *E. coli*.

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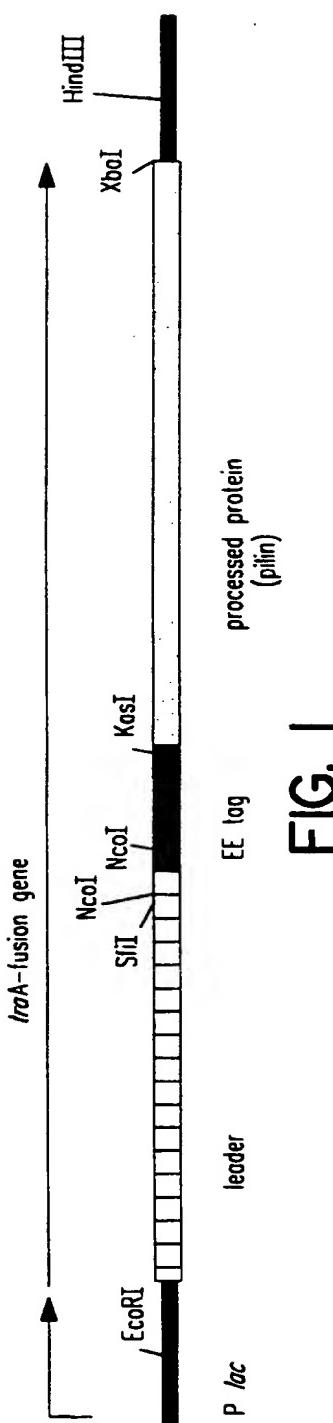


FIG. 1

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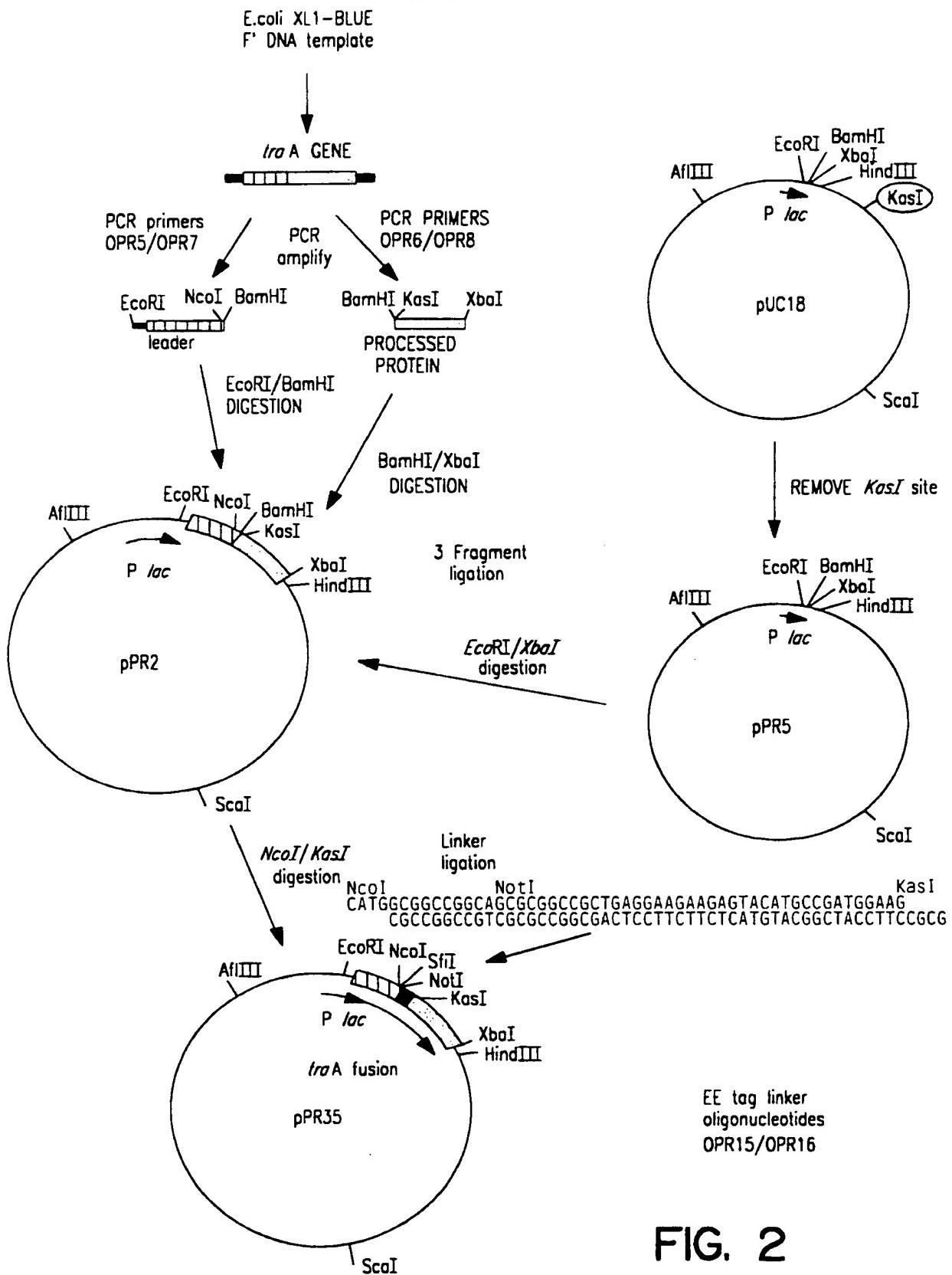


FIG. 2

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## PCR PRIMERS

traA LEADER FORWARD - OPR5

5'-GGGGGGAATTCTATCCGAAATTGAGGTAACCTTATG-3' (SEQ ID NO:1)

traA BACK - OPR6

5'-GGGGGTCTAGATTATCAGAGGCCAACGACGGCCATAAC-3' (SEQ ID NO:2)

traA LEADER BACK - OPR7

5'-GGGGGATCCCCATGGCCAGCTGCGGGAAAGAACATCATCAG-3' (SEQ ID NO:3)

traA GENE FORWARD - OPR8

5'-GGGGGATCCGGCGCCGCAGCAGTGGTCAGGACCTGATG-3' (SEQ ID NO:4)

TA1 HC FORWARD - JS135

5'-CACTGGCATGGCGAGGTTAGCTGCAGCAG-3' (SEQ ID NO:5)

TA1 HC BACK - JS134

5'-GCTGCCACCGCACCTGAGGAGACGGTGACTGAG-3' (SEQ ID NO:6)

TA1 LC FORWARD - JS133

5'-GGAGGCAGGGTCTGATATTGTGATGACTCAGGC-3' (SEQ ID NO:7)

TA1 LC BACK - JS153

5'-TTCATAGGCAGGGCACTAGTAGCMCGTTCAAGYTCCARC-3' (SEQ ID NO:8)

 $\alpha$ -CKMB HC FORWARD - JS155

5'-GCCGGCCATGGCCCAGGTBCARCTKMARSARTC-3' (SEQ ID NO:9)

 $\alpha$ -CKMB HC BACK - JS160

5'-GCTGCCACCGCACCTGMRGAGACDGTGASTGARG-3' (SEQ ID NO:10)

 $\alpha$ -CKMB LC FORWARD - JS148

5'-GGAGGCAGGGTCTGACATTGTGMTGWCACAGTC-3' (SEQ ID NO:11)

 $\alpha$ -CKMB LC BACK - JS154

5'-TTCATAGGCAGGGCACTAGTAGCMCGTTKATYTCCARC-3' (SEQ ID NO:12)

(GGGS)<sub>3</sub> LINKER PRIMERS - JS1375'-GGTGGCGGTGGCAGCGGGGTGGTGGTCCGGAGGCAGGCGGTTCT-3' (SEQ ID NO.13)

JS139

5'-AGAACCGCCCTCCGGAAGGAGGACCGGCCGCTGCCACCGCCACC-3' (SEQ ID NO:14)

## EE TAG OLIGONUCLEOTIDES

OPR15

5'-CATGGCGGCCGGCAGCGCGGGCCGTGAGGAAGAAGAGTACATGCCGATGGAAG-3' (SEQ II NO.15)

OPR16

5'-GCGCCTTCCATCGGCATGTACTCTTCTCCTCAGCGGCCGGCTGCCGGCCGC-3' (SEQ II NO.16)**FIG. 3**

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>EcoR1

---

GAAAC AGCTA TGACC ATGAT TACGA ATTCT ATCCG AAATT GAGGT AACTT ATG AAT GCT  
 CTTTG TCGAT ACTGG TACTA ATGCT TAAGA TAGGC TTAACTCCTA TTGAA TAC TTA CGA  
 M N A>

Plac            -10 REGION            traA RBS

---

GTT TTA AGT GTT CAG GGT GCT TCT GCG CCC GTC AAA AAG AAG TCG TTT TTT TCC  
 CAA AAT TCA CAA GTC CCA CGA AGA CGC GGG CAG TTT TTC TTC AGC AAA AAA AGG  
 V L S V Q G A S A P V K K K S F F S>

traA LEADER

---

AAA TTC ACT CGT CTG AAT ATG CTT CGC CTG GCT CGC GCA GTG ATC CCG GCT GCT  
 TTT AAG TGA GCA GAC TTA TAC GAA GCG GAC CGA GCG CAC TAG GGC CGA CGA  
 K F T R L N M L R L A R A V I P A A>

>Nco1      >Sfi1      >Not1

---

GTT CTG ATG ATG TTC TTC CCG CAG CTG GCC ATG GCG GCC GGC AGC GCG GCC GCT  
 CAA GAC TAC TAC AAG AAG GGC GTC GAC CGG TAC CGC CGG TCG CGC CGG CGA  
 V L M M F F P Q L A M A A G S A A A>

CLONING SITES

>Kas1

---

GAG GAA GAA GAG TAC ATG CCG ATG GAA GGC GCC GGC AGT GGT CAG GAC CTG  
 CTC CTT CTT CTC ATG TAC GGC TAC CTT CCG CGG CGG TCG TCA CCA GTC GAC CTG  
 E E E E Y M P M E G A G S S G Q D L>

EE TAG

FIG. 4A

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ATG	GCA	AGC	GGT	AAC	ACC	ACG	GTT	AAG	GCG	ACC	TTC	GGT	AAG	GAC	TCC	AGT	GTT
TAC	CGT	TCG	CCA	TTG	TGG	TGC	CAA	TTC	CGC	TGG	AAG	CCA	TTC	CTG	AGG	TCA	CAA
M	A	S	G	N	T	T	V	K	A	T	F	G	K	D	S	S	V>

**traA GENE**

GTT	AAA	TGG	GTT	GTT	CTG	GCT	GAA	GTT	CTG	GTC	GGT	GCT	GTC	ATG	TAC	ATG	ATG
CAA	TTT	ACC	CAA	CAA	GAC	CGA	CTT	CAA	GAC	CAG	CCA	CGA	CAG	TAC	ATG	TAC	TAC
V	K	W	V	V	L	A	E	V	L	V	G	A	V	M	Y	M	M>

**traA GENE (CONT.)**

ACC	AAA	AAC	GTC	AAG	TTC	CTG	GCC	GGT	TTT	GCC	ATC	ATC	TCT	GTA	TTT	ATT	GCT
TGG	TTT	TTG	CAG	TTC	AAG	GAC	CGG	CCA	AAA	CGG	TAG	TAG	AGA	CAT	AAA	TAA	CGA
T	K	N	V	K	F	L	A	G	F	A	I	I	S	V	F	I	A>

## &gt;XbaI

GTG	GTT	ATG	GCC	GTC	GTT	GGC	CTC	TGA	TAA	TCT	AGAGT	CGACC	TGCAG	GCATG			
CAC	CAA	TAC	CGG	CAG	CAA	CCG	GAG	ACT	ATT	AGA	TCTCA	GCTGG	ACGTC	CGTAC			
V	V	M	A	V	V	G	L	*	*	>	(SEQ ID NO:18)						

**STOP CODONS**

## &gt;Hind3

CAAGC	TTGGC	ACTGG	CCGTC	GTTTT	ACAAC	GTCGT	GACTG	GGAAA	ACCCCT								
GTTCG	AACCG	TGACC	GGCAG	CAAAA	TGTTG	CAGCA	CTGAC	CCTTT	TGGGA	(SEQ ID NO:17)							

**FIG. 4B**

Initial cloning scheme 6 / 20

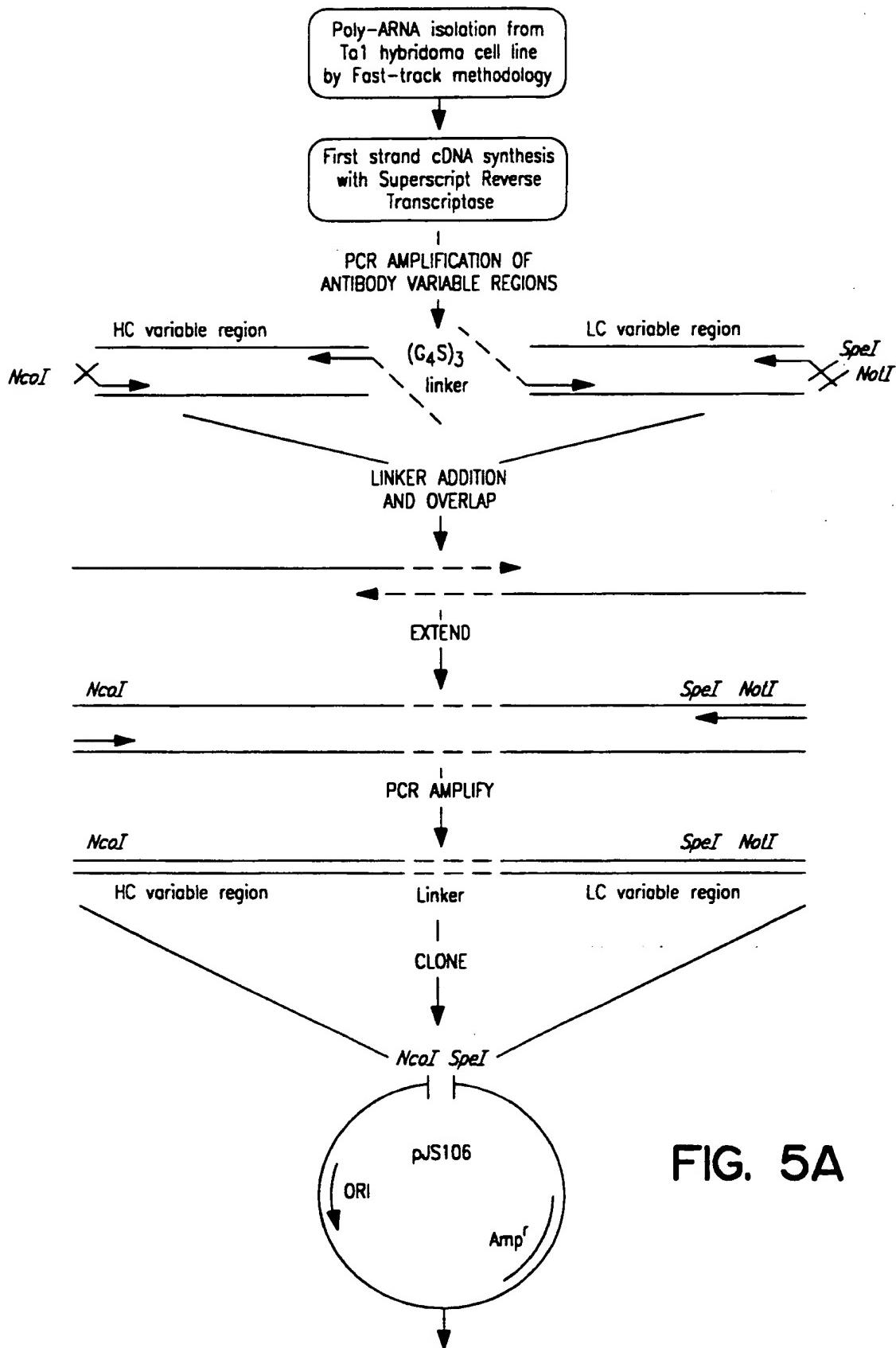


FIG. 5A

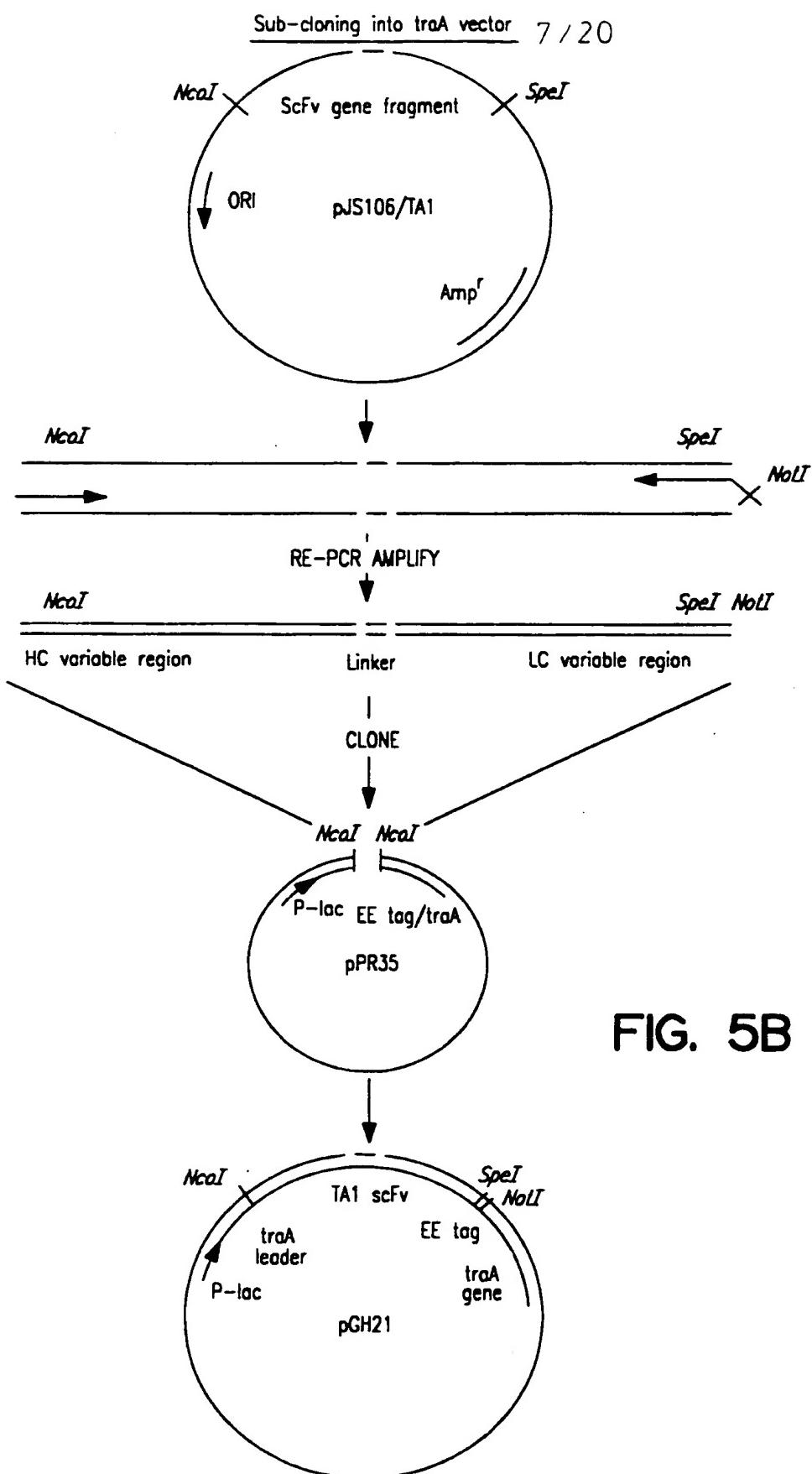


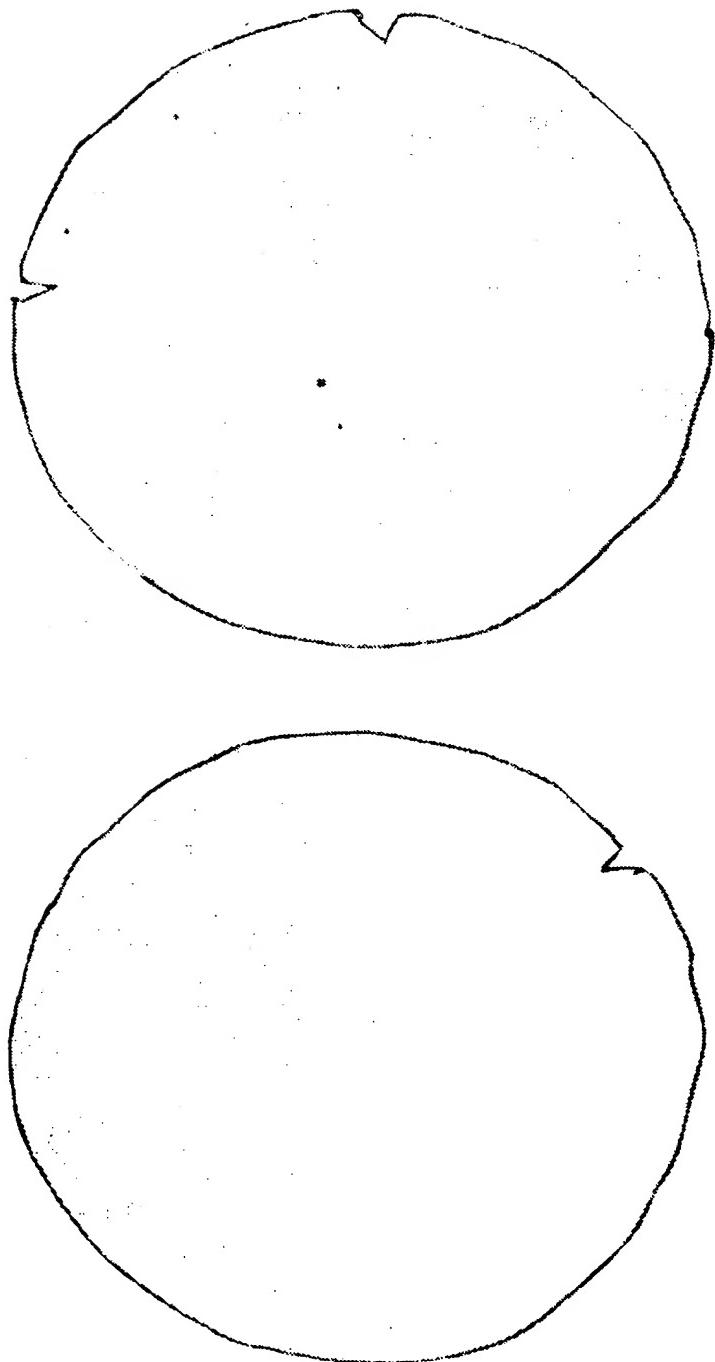
FIG. 5B

FIG. 6

GluGlu tag/trA FUSION PROTEIN EXPRESSION

NON-INDUCED

INDUCED



BACTERIAL COLONIES PROBED WITH ANTI-GluGlu tag ANTIBODIES

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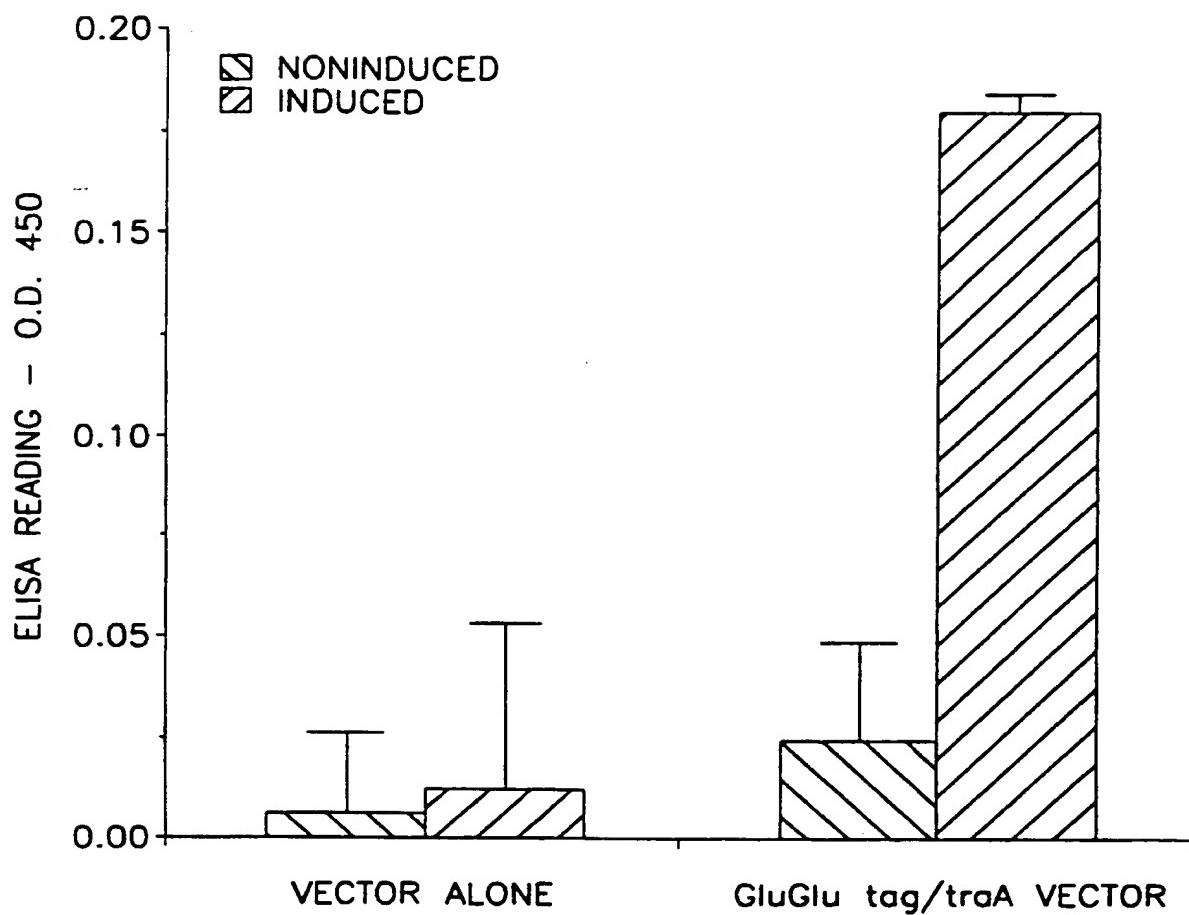


FIG. 7

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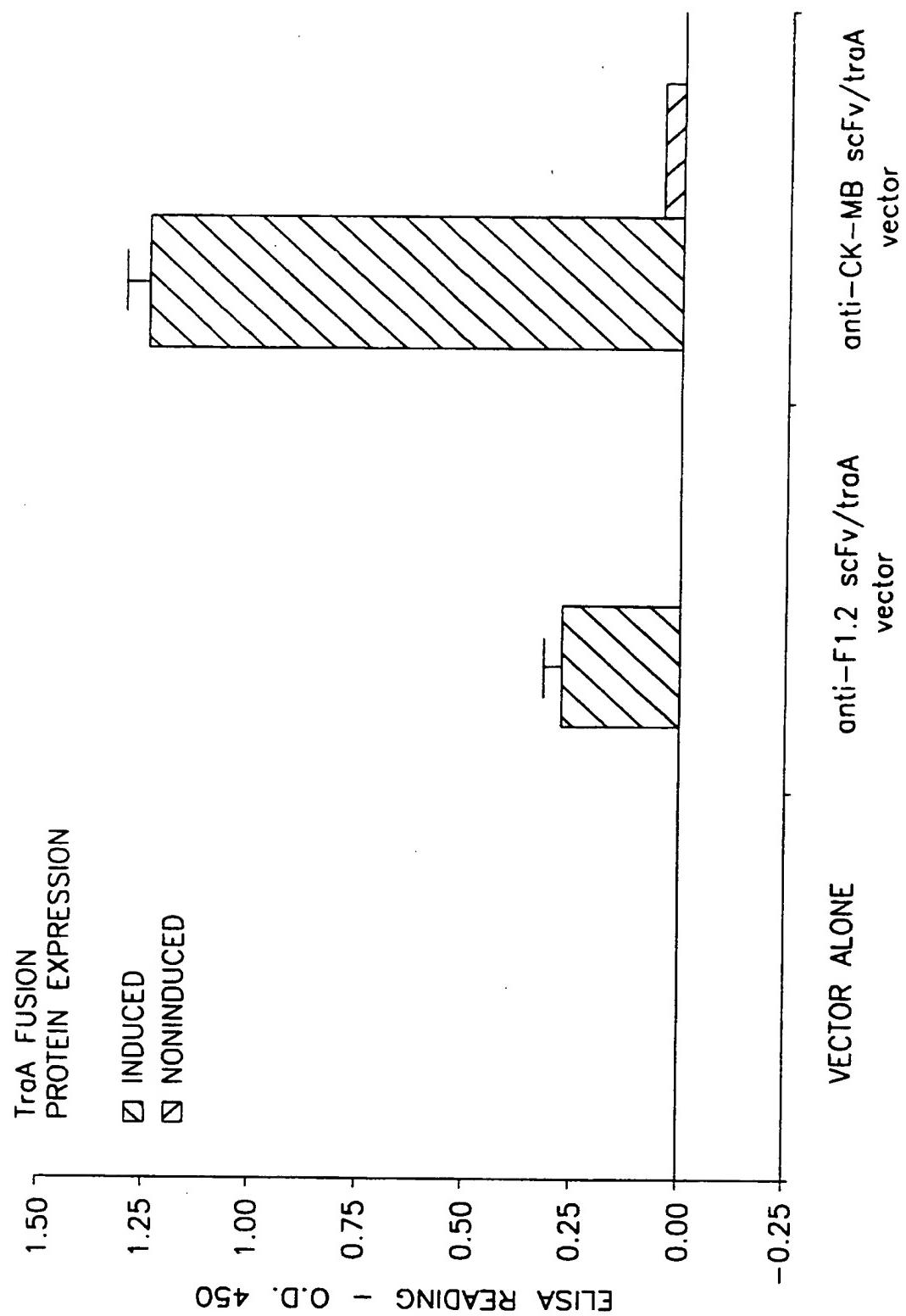
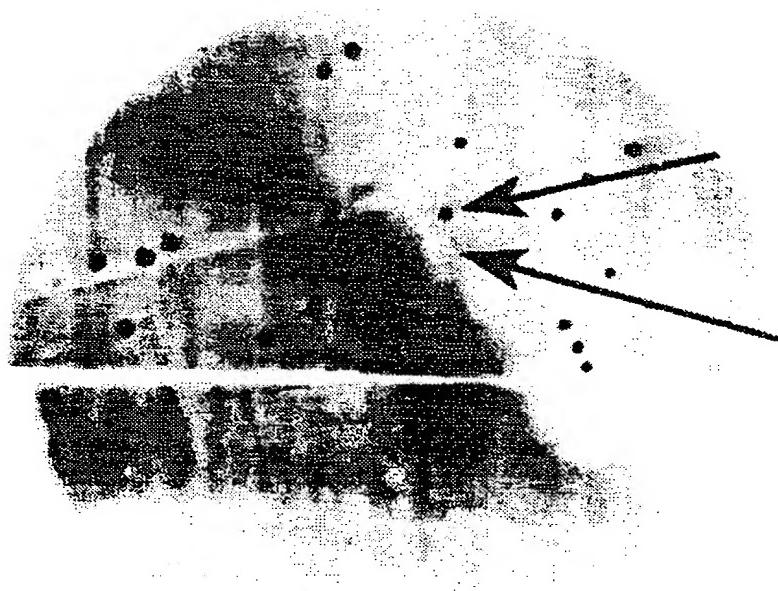


FIG. 8

**FIG. 9**

ScFv-trA FUSION PROTEIN EXPRESSION  
NON-INDUCED      INDUCED



SIGNAL FOR NEGATIVE CONTROL COLONY  
EXPRESSING ANTI-F1.2 scFv-TrA  
FUSION PROTEIN

SIGNAL FOR COLONY EXPRESSING  
ANTI-CK-MB scFv-TrA  
FUSION PROTEIN

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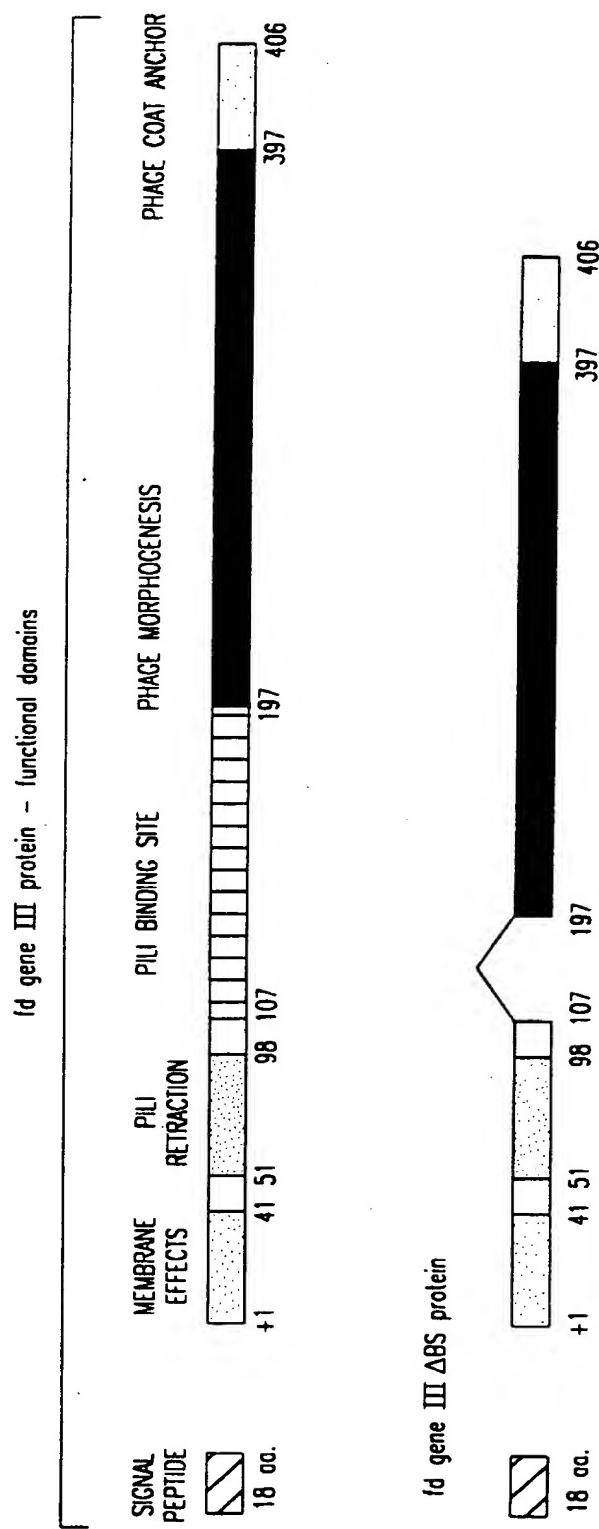
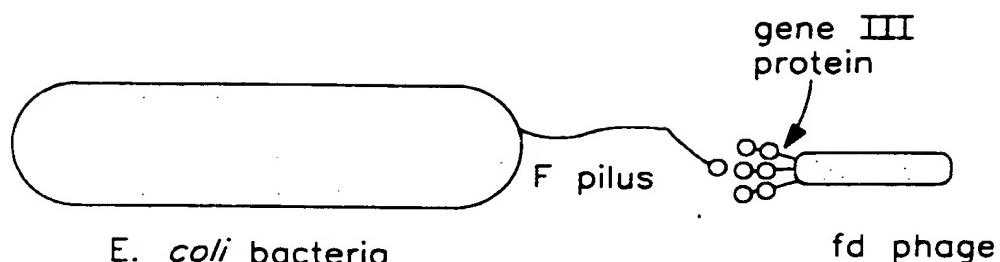


FIG. 10

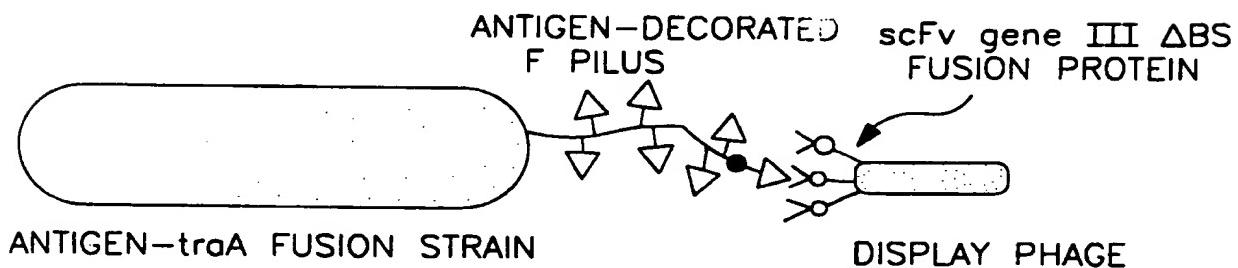
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## NORMAL INFECTION



- 1) BINDING OF PHAGE TO THE TIP OF THE PILUS
- 2) DEPOLYMERIZATION OF PILUS
- 3) ENTRY OF THE PHAGE INTO THE CELL

## ANTIGEN/ANTIBODY DRIVEN INFECTION



NORMAL GENE III PROTEIN/PILIN INTERACTION IS  
REPLACE BY ANTIGEN/ANTIBODY INTERACTION

**FIG. 1**

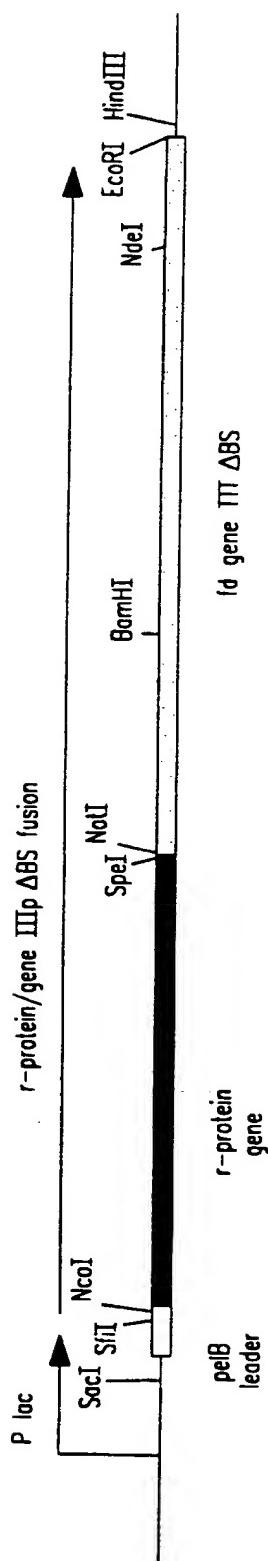
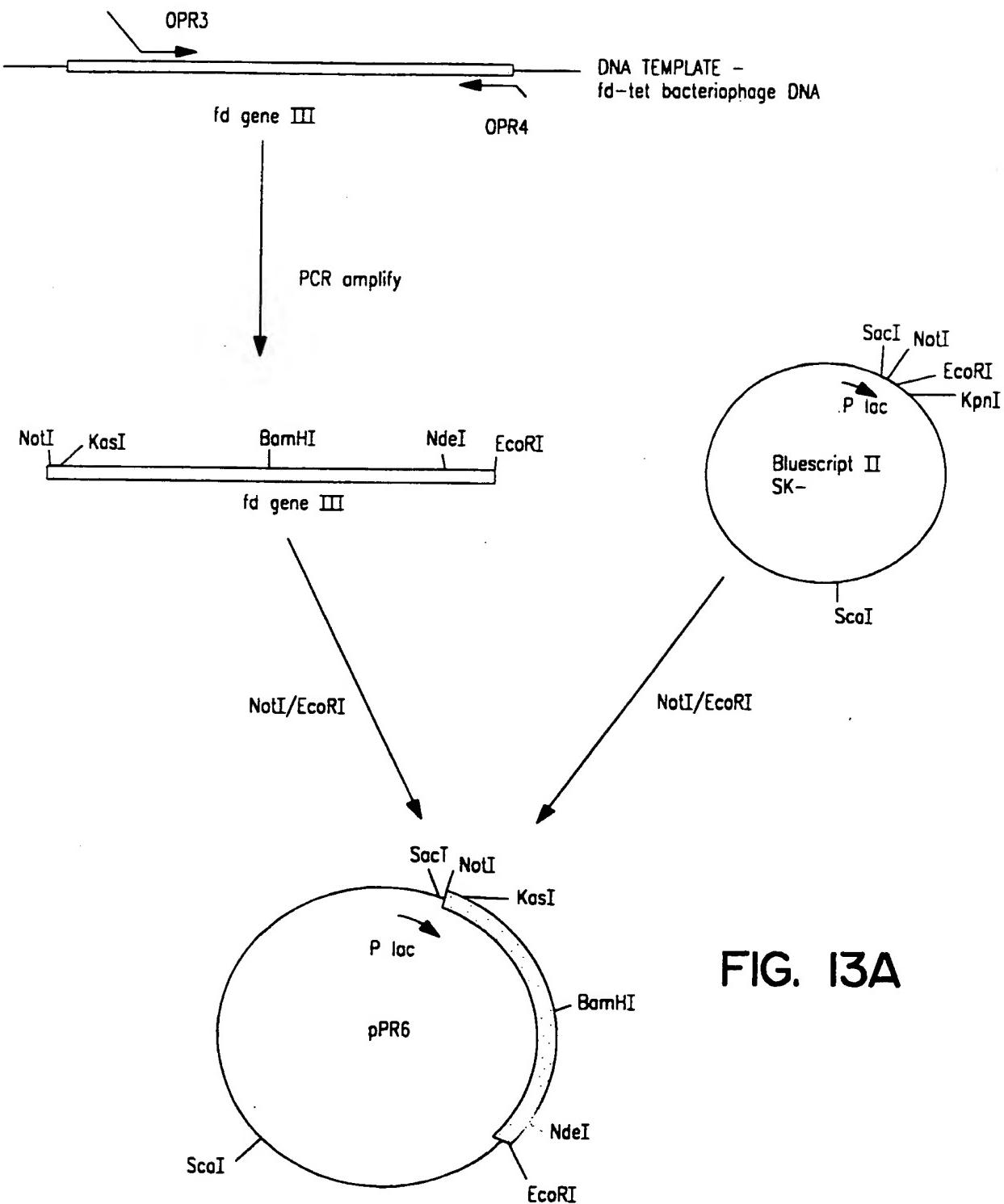
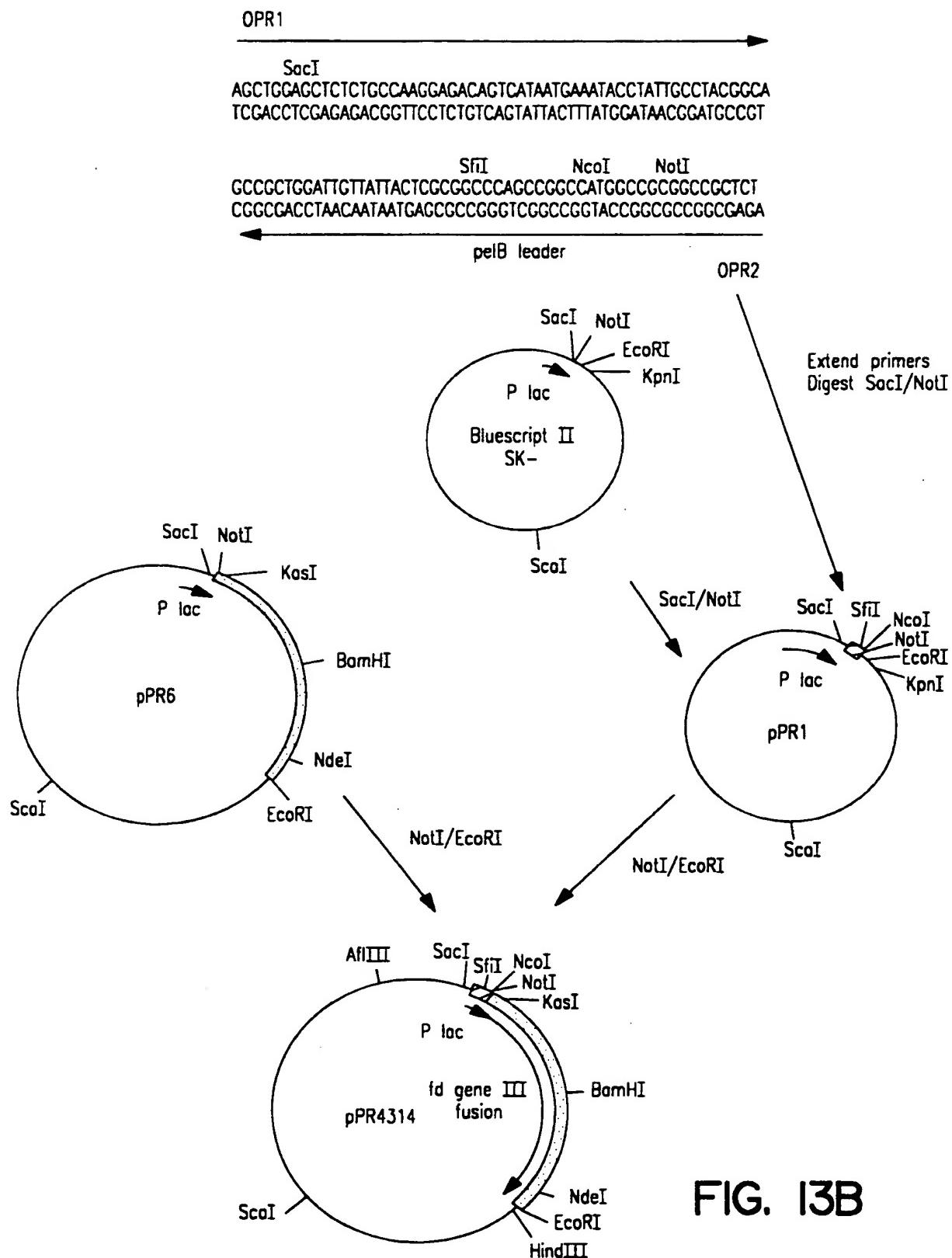


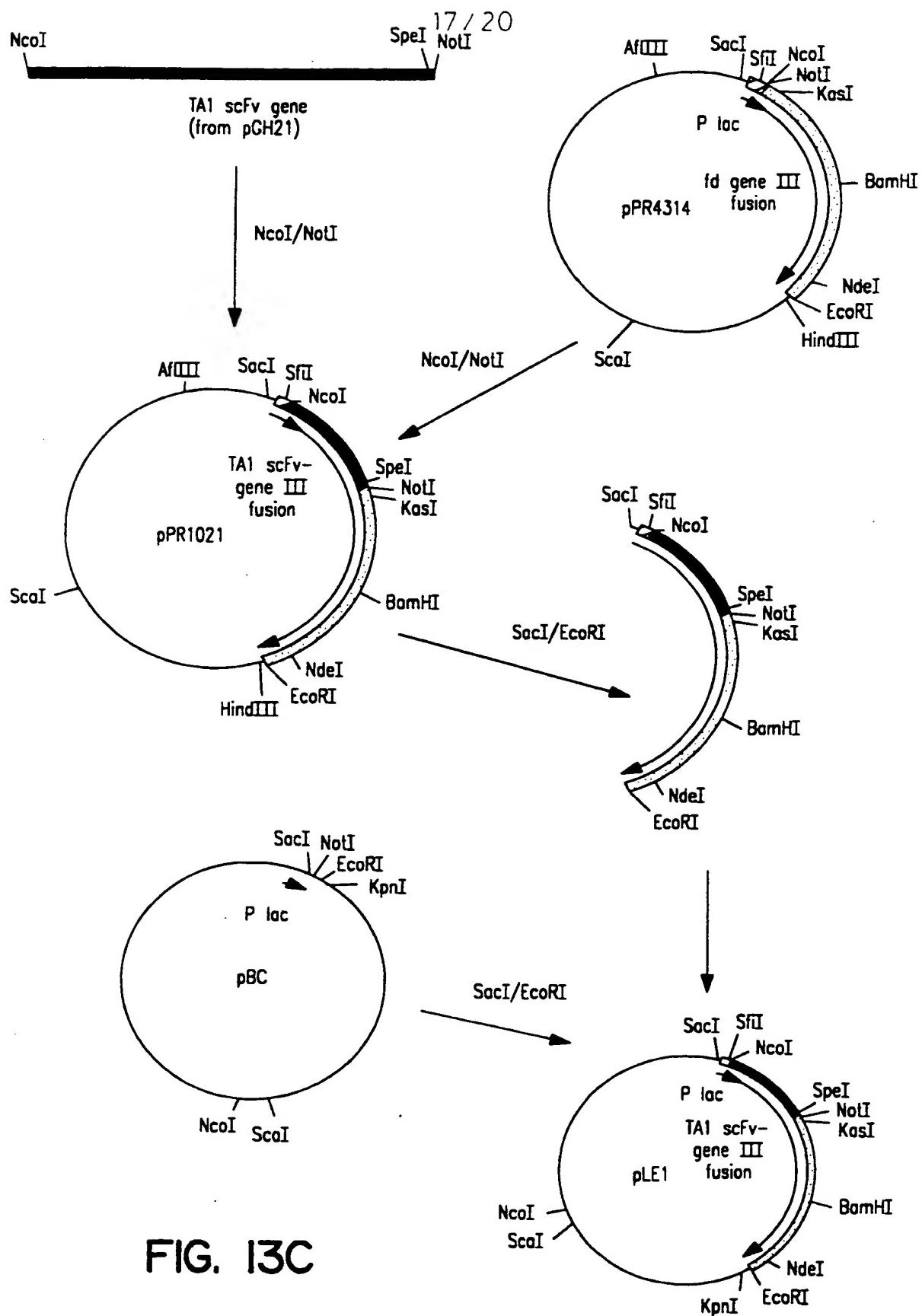
FIG. 12

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**FIG. 13C**

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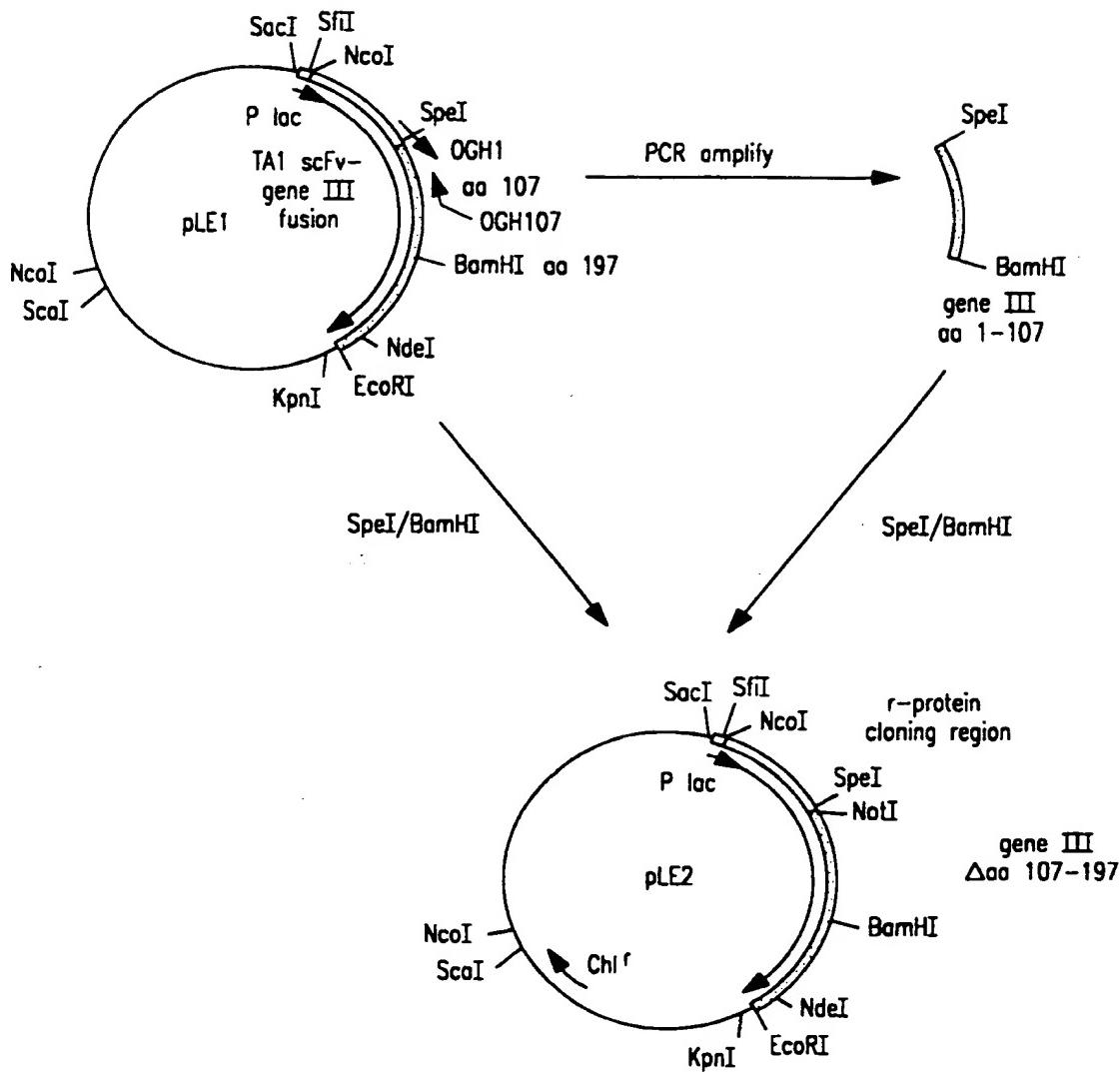


FIG. 13D

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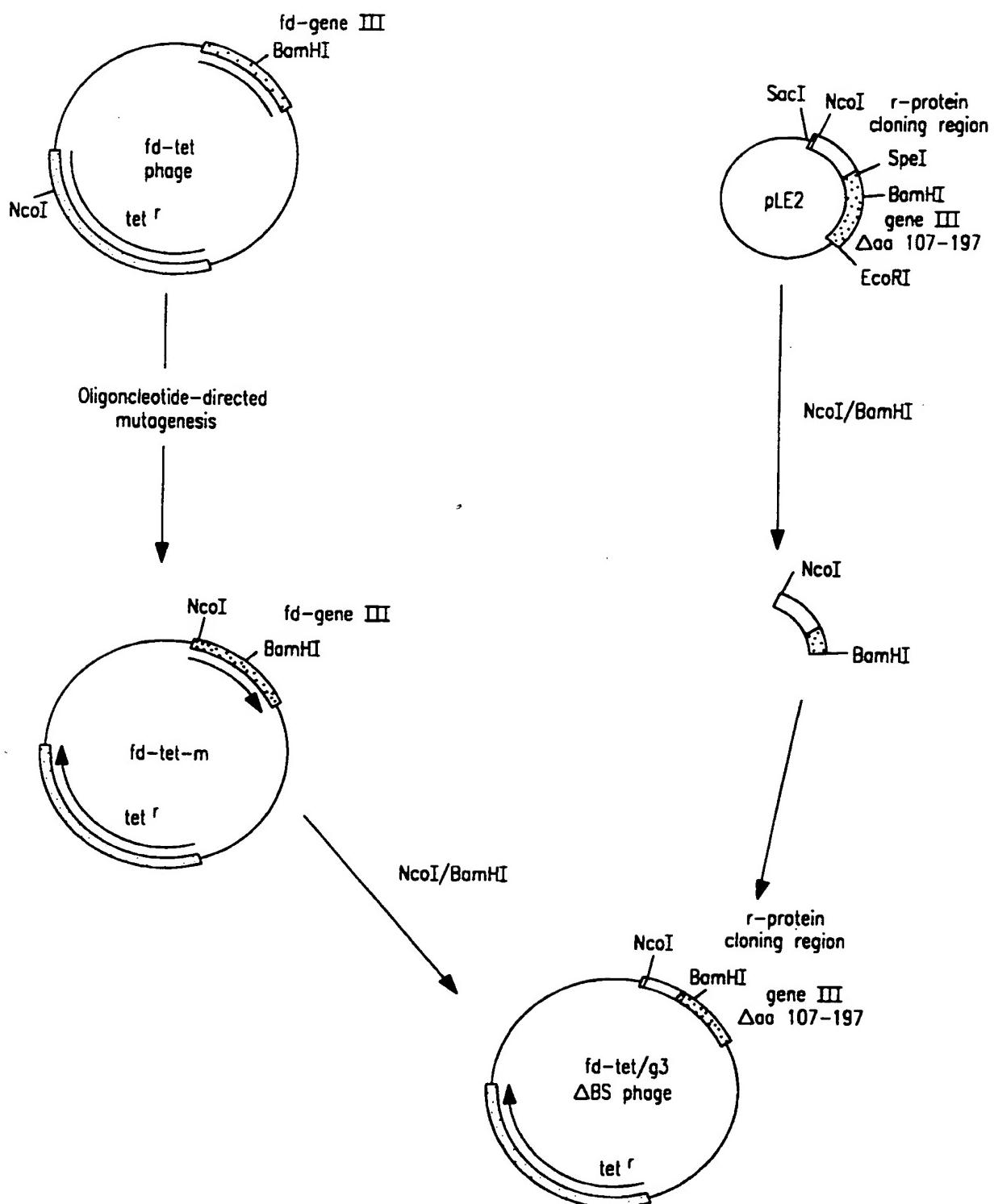
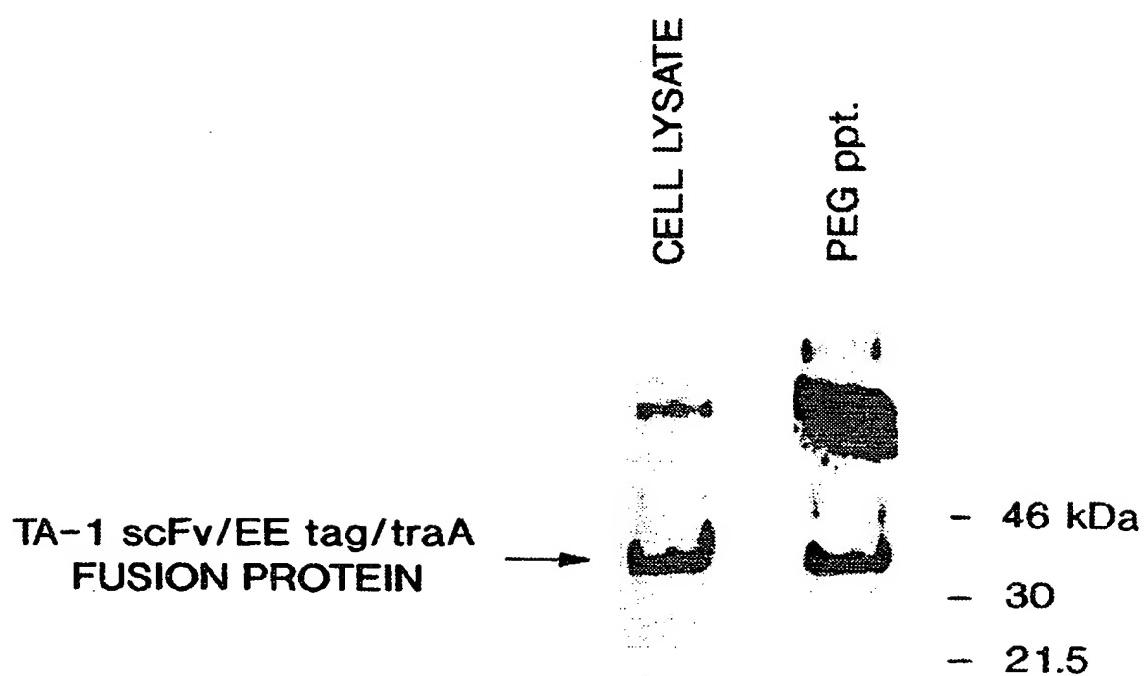


FIG. 14

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FIG. 15



## INTERNATIONAL SEARCH REPORT

Interr. Application No  
PCT/US 95/07541

A. CLASSIFICATION OF SUBJECT MATTER					
IPC 6	C12N15/10	C12N15/62	C12N15/70	C12N1/21	C07K14/245
	C07K16/00	C12Q1/70	C12Q1/02	G01N33/53	G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C12Q C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 264 150 (SOLVAY) 20 April 1988 the whole document	1-3,9 10,12,13
Y	---	
Y	SCIENCE, vol. 244, 7 April 1989 AAAS, WASHINGTON, DC, US, pages 70-72, S.M.C. NEWTON ET AL. 'Immune response to cholera toxin epitope inserted in Salmonella flagellin' the whole document	1-3,9, 10,12,13
Y	DE,C,41 22 598 (DEUTSCHES KREBSFORSCHUNGSZENTRUM) 30 July 1992 the whole document	1-3,9, 10,12,13
	---	
	-/-	

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

## \* Special categories of cited documents :

- 'A' document defining the general state of the art which is not considered to be of particular relevance
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- 'P' document published prior to the international filing date but later than the priority date claimed

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- 'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- '&' document member of the same patent family

Date of the actual completion of the international search

2 October 1995

Date of mailing of the international search report

0 6 . 1 0 . 9 5

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Hornig, H

## INTERNATIONAL SEARCH REPORT

Interns | Application No  
PCT/US 95/07541

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,93 18163 (THE ROCKEFELLER UNIVERSITY) 16 September 1993 the whole document ---	1,9,10, 12,13
A	NATURE, vol. 348, 6 December 1990 MACMILLAN JOURNALS LTD., LONDON,UK, pages 552-554, J. MCCAFFERTY ET AL. 'Phage antibodies: filamentous phage displaying antibody variable domains' the whole document ---	14-29
A	WO,A,91 18980 (CETUS CORPORATION) 12 December 1991 the whole document ---	14-29
A	WO,A,91 19818 (AFFYMAX TECHNOLOGIES) 26 December 1991 the whole document ---	14-29
A	WO,A,93 10214 (GEORGIOS GEORGE) 27 May 1993 cited in the application the whole document ---	1-13
A	J. BACTERIOL., vol. 160, no. 1, October 1984 AM. SOC. MICROBIOL., BALTIMORE, US;, pages 395-401, L.S. FROST ET AL. 'DNA sequence of the F traALE region that includes the gene for F pilin' cited in the application the whole document ---	14-29
A	J. BACTERIOL., vol. 164, no. 12, December 1985 AM. SOC. MICROBIOL., BALTIMORE, US;, pages 1238-1247, L.S. FROST ET AL. 'Characterization and sequence analysis of pilin from F-like plasmids' cited in the application the whole document ---	14-29
P,Y	FR,A,2 699 538 (INST NATIONAL DE LA RECHERCHE AGRONOMIQUE) 24 June 1994 the whole document -----	1-3,9, 10,12,13

## INTERNATIONAL SEARCH REPORT

Intern. Application No.  
PCT/US 95/07541

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0264150	20-04-88	FR-A-	2605017	15-04-88
		FR-A-	2607149	27-05-88
		DE-A-	3783425	18-02-93
		JP-A-	63185394	30-07-88
DE-C-4122598	30-07-92	WO-A-	9301287	21-01-93
		EP-A-	0547200	23-06-93
		JP-T-	6501395	17-02-94
WO-A-9318163	16-09-93	CA-A-	2131995	16-09-93
		EP-A-	0646176	05-04-95
WO-A-9118980	12-12-91	AU-A-	8081491	31-12-91
		EP-A-	0600866	15-06-94
WO-A-9119818	26-12-91	AU-A-	8285291	07-01-92
		EP-A-	0535151	07-04-93
		JP-T-	5508321	25-11-93
		US-A-	5432018	11-07-95
WO-A-9310214	27-05-93	US-A-	5348867	20-09-94
		CA-A-	2123676	27-05-93
FR-A-2699538	24-06-94	AU-B-	6808794	19-07-94
		WO-A-	9414967	07-07-94